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**Pharmacological characterisation of selective Y4 and dual Y2/Y4 receptor agonists novel targets for putative anti-obesity therapies**

Jolly, Simmi

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**Author:** Simmi Jolly

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**Pharmacological characterisation of selective Y<sub>4</sub> and dual Y<sub>2</sub>/Y<sub>4</sub> receptor agonists:  
novel targets for putative anti-obesity therapies**

A thesis submitted in partial satisfaction of the requirement for the degree of Doctor of  
Philosophy

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Wolfson Centre for Age-Related Diseases  
King's College London  
University of London  
2013

## Abstract

The GIPIO (Gastrointestinal Peptides in Obesity) collaboration was set up with EU (FP7) funding in 2008, with the aim to develop a series of peptide analogues for the treatment of obesity. Based on the dual  $Y_2/Y_4$  agonist, Obinepitide ( $[Q^{34}]hPP$ ), and the  $Y_4$  agonist, TM30339 ( $hPP_{2-36}$ ), novel peptides were developed and subsequently modified by either PEGylation or lipidation to improve their relatively short half-life and pharmacokinetic profile. This thesis presents a detailed study of the *in vitro* pharmacological data performed to aid the progression of the most promising peptide candidates. An assessment of  $Y_2Y_4$  potency and activity was made, using optimised  $Y_4$  (human colonic epithelial monolayers) and  $Y_2Y_4$  (human colon mucosae) bioassays, where preparations were mounted in Ussing chambers and changes in short-circuit current ( $I_{sc}$ ) recorded. In addition, receptor specificity of the most promising agonists was dissected using  $Y_1$  and  $Y_2$  antagonists. Furthermore, an assessment of  $Y_4$  receptor desensitisation was made by application of a subsequent addition of the native hormone, hPP. With a lysine linker at position 30 or 22, chemical modification of Obinepitide with either PEGylation or Palmitoylation resulted in prolonged reductions in  $I_{sc}$  in both preparations compared with the transient response seen with their respective predecessors. Increasing the PEG size resulted in greater reductions in  $I_{sc}$ , though a PEG22 modification resulted in a loss of functional activity. Mid to long chain lipidation of TM30339 (lauric acid and palmitic acid) also resulted in sustained reductions in  $I_{sc}$ . Interestingly, lipidation with a short chain fatty acid (caprylic acid) caused a biphasic response, with an initial transient drop in  $I_{sc}$  followed by a sustained anti-secretory response. Importantly, PEG and lipid side chains had no effect upon  $I_{sc}$ . Pre-treatment of either human mucosa or epithelial monolayers with PEG2-, or 5-ylated Obinepitide did not cause significant subsequent  $Y_4$  desensitisation, an effect that was greater with the larger PEGylated peptide. Contrary to this, long chain lipidated peptides seemed to facilitate rapid and sustained desensitisation, revealed by the loss of further  $Y_4$  signalling even after nM lipidated agonist. This divergence in receptor signalling was also observed in fluorescent imaging studies performed by our collaborators. The data presented herein provides the first functional evidence of prolonged activity at the  $Y_2$  and  $Y_4$  receptors and these modified peptides have the potential to act for longer *in vivo* as anti-obesity treatments.

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## Abbreviations

<b>5-HT</b>	5-hydroxytryptamine
<b>α-MSH</b>	α-melanocyte stimulating hormone
<b>a</b>	avian
<b>AA</b>	arachidonic acid
<b>AC</b>	adenylyl cyclase
<b>Ach</b>	acetylcholine
<b>AE</b>	anion exchanger
<b>AgRP</b>	agouti-related protein
<b>ANOVA</b>	analysis of variance
<b>AP2</b>	adaptor protein 2
<b>ARC</b>	arcuate
<b>ASE</b>	acid saline extract
<b>β<sub>2</sub>-AR</b>	β <sub>2</sub> -adrenergic receptor
<b>b</b>	bovine
<b>BIBO3304</b>	( <i>R</i> )- <i>N</i> -[[4-(aminocarbonylaminomethyl)-phenyl]methyl]- <i>N</i> <sup>2</sup> -(diphenylacetyl)-argininamide trifluoroacetate
<b>BIBP3226</b>	( <i>R</i> )- <i>N</i> <sup>2</sup> -(diphenylacetyl)- <i>N</i> -[(4-hydroxyphenyl)methyl]-argininamide

<b>BIIE0246</b>	( <i>S</i> )- <i>N</i> <sup>2</sup> -[[1-[2-[4-[( <i>R,S</i> )-5,11-dihydro-6(6 <i>h</i> )-oxodibenz[ <i>b,e</i> ]azepin-11-yl]-1-piperazinyl]-2-oxoethyl] cyclopentyl] acetyl]- <i>N</i> -[2-[1,2-dihydro-3,5(4 <i>H</i> )-dioxo-1,2-diphenyl-3 <i>H</i> -1,2, 4-triazol-4-yl]ethyl]-argininamid
<b>BMI</b>	body mass index
<b>BN</b>	bombesin
<b>BRET</b>	bioluminescence resonance energy transfer
<b>C-</b>	carboxyl
<b>cAMP</b>	cyclic adenosine monophosphate
<b>CART</b>	cocaine and amphetamine-regulated transcript
<b>CCK</b>	cholecystokinin
<b>CF</b>	cystic fibrosis
<b>CFP</b>	cyan fluorescent protein
<b>CFTR</b>	cystic fibrosis transmembrane regulator
<b>CFTR<sup>-/-</sup></b>	cystic fibrosis transmembrane regulator knockout
<b>CGRP</b>	calcitonin gene-related peptide
<b>Ch</b>	chicken
<b>CHO</b>	Chinese hamster ovary
<b>CNS</b>	central nervous system
<b>Col-1</b>	Colony-1
<b>Col-6</b>	Colony-6
<b>Col-24</b>	Colony-24
<b>CPE</b>	carboxypeptidase E
<b>CPP</b>	cell penetrating peptide
<b>DAMGO</b>	[D-Ala <sup>2</sup> ,N-MePhe <sup>4</sup> , Gly-ol]enkephalin
<b>DIO</b>	diet-induced obese
<b>DMEM</b>	Dulbecco's modified Eagle's medium
<b>DMSO</b>	dimethyl sulphoxide
<b>DPPIV</b>	dipeptidyl peptidase IV
<b>DRA</b>	downregulated in adenoma
<b>ENaCs</b>	epithelial Na <sup>+</sup> channels
<b>ENS</b>	enteric nervous system
<b>ER</b>	endoplasmic reticulum
<b>EYFP</b>	enhanced yellow fluorescent protein
<b>FCS</b>	foetal calf serum

<b>FDA</b>	Food and Drug Administration
<b>FITC</b>	fluorescein isothiocyanate
<b>FRET</b>	fluorescent resonance energy transfer
<b>GDP</b>	guanosine-5'-diphosphate
<b>GFP</b>	green fluorescent protein
<b>GHS</b>	growth hormone secretagogue
<b>GI</b>	gastrointestinal
<b>GIP</b>	gastric inhibitory polypeptide
<b>GIPIO</b>	Gastrointestinal Peptides in Obesity
<b>Gln</b>	glutamine
<b>GLP-1</b>	glucagon-like peptide-1
<b>GLP-2</b>	glucagon-like peptide-2
<b>GPCR</b>	G protein-coupled receptor
<b>GTP</b>	guanosine-5'-triphosphate
<b>GRK</b>	G protein-coupled receptor kinase
<b>h</b>	human
<b>HCA</b>	human colonic adenocarcinoma
<b>HEK</b>	human embryonic kidney
<b>HPLC</b>	high performance liquid chromatography
<b>IBMX</b>	isobutyl-1-methyl-xanthine
<b>IK</b>	intermediate conductance K <sup>+</sup>
<b><i>i.p.</i></b>	intraperitoneal
<b>IP<sub>3</sub></b>	inositol triphosphate
<b>IPAN</b>	intrinsic primary afferents
<b>Isc</b>	short-circuit current
<b><i>i.v.</i></b>	intravenous
<b>KH</b>	Kreb's-Henseleit
<b>KO</b>	knock out
<b>LHA</b>	lateral hypothalamic area
<b>m</b>	mouse
<b>MALDI-TOF-MS</b>	matrix assisted laser desorption/ionisation-time of flight mass spectrometry
<b>MAPK</b>	mitogen activated protein kinase
<b>MCH</b>	melanin-concentrating hormone
<b>MCR</b>	melanocortin receptor

<b>N-</b>	Nitrogen aminoterminal
<b>NA</b>	noradrenaline
<b>NEP</b>	neutral endopeptidase
<b>NK</b>	neurokinins
<b>NKCC1</b>	Na <sup>+</sup> /K <sup>+</sup> /2Cl <sup>-</sup> co-transporter
<b>NO</b>	nitric oxide
<b>NOS</b>	nitric oxide synthase
<b>NPH</b>	Neutral Protamine Hagedorn
<b>NPY</b>	neuropeptide Y
<b>NTS</b>	nucleus tractus solitarius
<b><i>ob/ob</i></b>	obese
<b>p</b>	porcine
<b>PACAP</b>	pituitary adenylyl cyclase activating peptide
<b>PC</b>	prohormone convertase
<b>PEG</b>	polyethylene glycol
<b>PGE2</b>	prostaglandin E2
<b>PKA</b>	protein kinase A
<b>POMC</b>	pro-opiomelanocortin
<b>PP</b>	pancreatic polypeptide
<b>Pro</b>	proline
<b>PTH</b>	parathyroid hormone
<b>PTHrP</b>	paracrine acting PTH-related protein
<b>PVN</b>	paraventricular nucleus
<b>PYY</b>	peptide YY
<b>QRT-PCR</b>	quantitative reverse transcriptase-polymerase chain reaction
<b>r</b>	rat
<b>rFVIIa</b>	factor VIIa
<b>rh</b>	rhesus
<b>RLUC</b>	<i>Renilla</i> luciferase
<b>S1P1</b>	sphingosine-1-phosphate receptor 1
<b>SCID</b>	Severe Combined Immunodeficiency Disease
<b>sCT</b>	salmon calcitonin
<b>Som</b>	somatostatin
<b>SRIF</b>	somatotropin release-inhibiting factor
<b>TAMRA</b>	tetramethylrhodamine

<b>TER</b>	transepithelial resistance
<b>TF</b>	tissue factor
<b>TM</b>	transmembrane
<b>TSH</b>	thyroid stimulating hormone
<b>TSHR</b>	thyroid stimulating hormone receptor
<b>TTX</b>	tetrodotoxin
<b>Tyr</b>	tyrosine
<b>UK 14,304</b>	5-bromo- <i>N</i> -(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine
<b>VAS</b>	visual analogue scales
<b>VIP</b>	vasoactive intestinal polypeptide
<b>Vt</b>	transepithelial voltage potential
<b>WT</b>	wild type
<b>Y</b>	Tyr
<b>Y<sub>1</sub><sup>-/-</sup></b>	Y <sub>1</sub> receptor knockout
<b>Y<sub>2</sub><sup>-/-</sup></b>	Y <sub>2</sub> receptor knockout
<b>Y<sub>4</sub><sup>-/-</sup></b>	Y <sub>4</sub> receptor knockout



### **Publications- citable abstracts**

N Jolly, C Elling, P Little, LO Gerlach TW Schwartz, HM Cox (2010). Functional activity of Y<sub>2</sub>/Y<sub>4</sub> receptor agonists for the treatment of obesity. *Br J Pharmacol*.

URL: <http://www.pa2online.org/abstracts/vol8issue1abst072P.pdf>

N Jolly, R Tolhurst, A Beck-Sickinger, TW Schwartz, HM Cox (2010). Ghrelin receptor expression and activities in the mouse gastrointestinal tract. *Br J Pharmacol*.

URL: <http://www.pa2online.org/abstracts/vol8issue1abst074P.pdf>

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## **1 INTRODUCTION**

## **1.1 The pancreatic polypeptide family**

### **1.1.1 Discovery of PP, PYY and NPY**

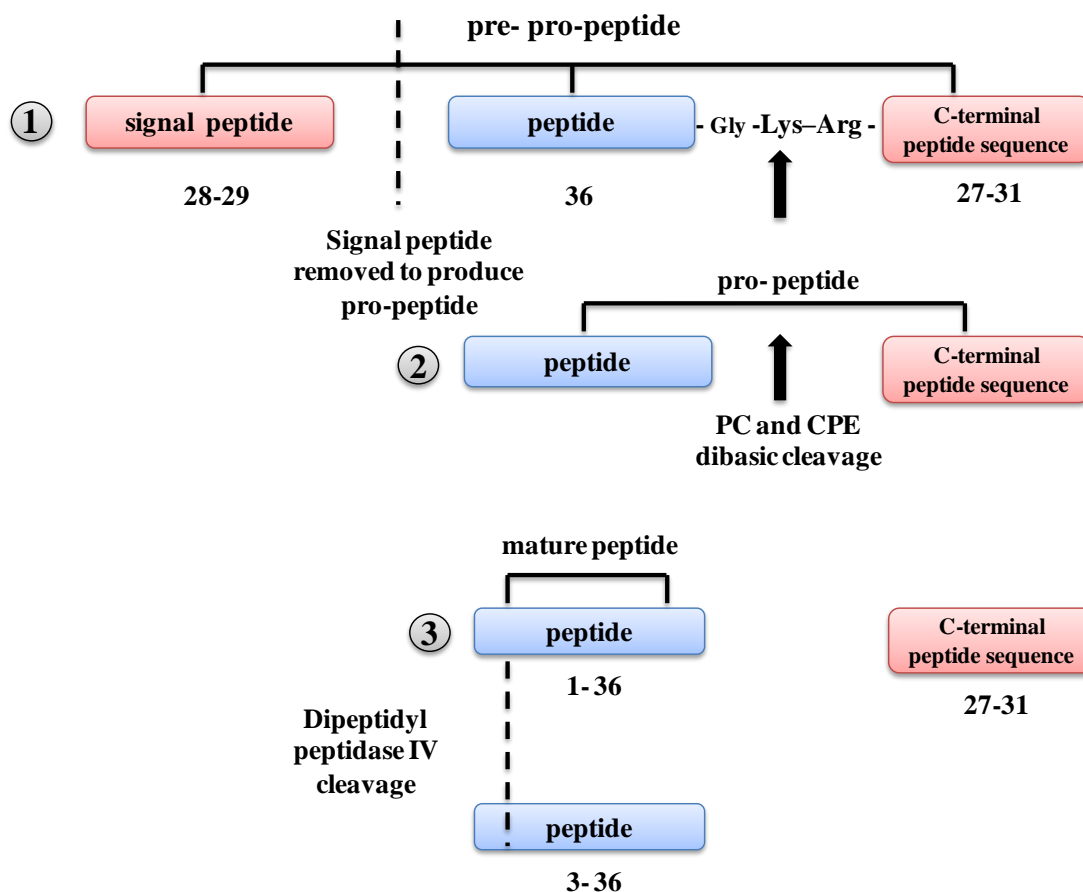
The circulating hormones pancreatic polypeptide (PP), peptide YY (PYY), the carboxyl (C)-terminal fragment PYY(3-36) and the neurotransmitter neuropeptide Y (NPY) all belong to the PP family, and in their full length form, consist of 36 amino acids. PP, the first of these peptides to be identified, was recognised as a by-product of insulin purification in chicken pancreas (Kimmel *et al.*, 1968), followed by successful isolation of the peptide by the same group (Kimmel *et al.*, 1975). PP immunoreactivity was subsequently measured in human pancreatic cells (Larsson *et al.*, 1975), in addition to mouse, rat (Larsson *et al.*, 1976) and a number of other mammalian, avian, reptilian, and amphibian species (Lundell *et al.*, 2000).

It was twelve years before a second peptide with sequence homology to that of PP was isolated, from the pig intestine (Tatemoto and Mutt, 1980). With the use of an innovative isolation technique, Tatemoto and Mutt characterised a 36-amino acid polypeptide possessing a tyrosine (Tyr) at both the N- and the C-terminal ends. It was these Tyr (Y) amino acids that led to the peptide being named peptide YY, or PYY. Using the same technique, subsequent studies in porcine (p) brain extracts led to the discovery and isolation of NPY (Tatemoto *et al.*, 1982), which possessed a structure similar to that of PP and PYY. Degradation of the PP family members (excluding PYY in non-mammals) by the dipeptidyl peptidase IV (DPPIV) enzyme results in the C-terminal fragments of PYY and NPY (Mentlein *et al.*, 1993); the enzyme cleaves the first two N-terminal Tyr and proline (Pro) residues generating the products PYY(3-36) and NPY(3-36). Gastrointestinal (GI) DPPIV mRNA expression levels were shown to be most abundant in the human distal small intestine, with second highest expression levels found in the colon (Darmoul *et al.*, 1994).

### **1.1.2 Synthesis of PP, PYY and NPY**

Synthesis of PP, PYY and NPY occurs in the endoplasmic reticulum (ER) where these peptides are generated as large precursor molecules. These precursor molecules are subsequently translocated to the Golgi-apparatus following post-translational modification, whereby they undergo sorting in the trans-Golgi network. In the pre-pro-peptide form, the mature peptides are flanked by a 28-29 amino acid N-terminal signal peptide, and a 27-31 amino acid C-terminal peptide sequence, connected to the mature peptide sequence by a glycine-lysine-arginine cleavage sequence. The signal peptide is

then removed to produce the pro-peptide form (Boel *et al.*, 1984; Leiter *et al.*, 1984; Leiter *et al.*, 1987). Following hydrolysis of the pro-peptide form at the cleavage site, the C-terminal peptide sequence is separated from the mature peptide, as illustrated in Figure 1.1.

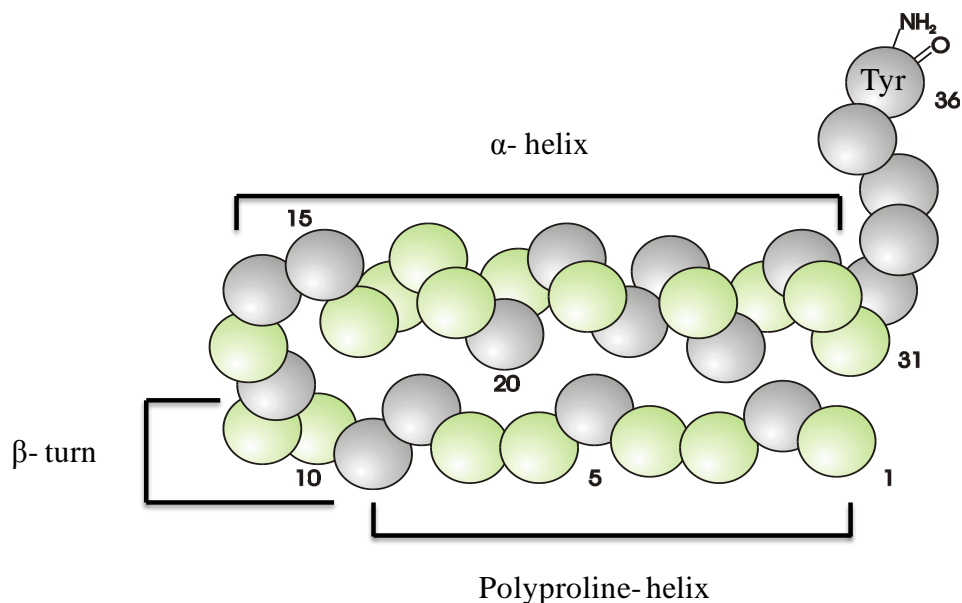


**Figure 1.1 The post-translational modification of PP, PYY and NPY.** The peptide is generated as a large precursor consisting of the pre-pro-peptide and then flanked by a C-peptide and a signal peptide. After enzymatic cleavage of the signal peptide, a pro peptide is generated (1). Enzymes including carboxypeptidase E (CPE) and prohormone convertase (PC) cleave the pro-peptide at the dibasic site (2), thereby generating a mature peptide (3), which can then be truncated to PYY(3-36) by the DPPIV enzyme.

### 1.1.3 The PP-fold structure of PP, PYY and NPY

Each member of the PP family has the key residues required to adopt the PP-fold, which comprises a polyproline helix and an interdigitating amphipathic  $\alpha$ -helix and a  $\beta$ -turn (Figure 1.2). The structure of turkey PP was first elucidated in 1981 with the use of X-ray crystallography (Blundell *et al.*, 1981), which also highlighted an amidated C-terminal Tyr residue as part of the structure. Despite being the first peptide to be discovered, PP is predicted to be the most recent member of the NPY family in terms of evolution and was thought to have arisen by the duplication of the PYY gene in early

tetrapods (amphibians, birds, reptiles and mammals; Larhammar *et al.*, 1993). More recently, Larhammar and Bergqvist (2013) provided evidence for the occurrence of this duplication very early in the sarcopterygian lineage, before the origin of tetrapods, by demonstrating PYY gene duplication in the West Indian Ocean coelacanth (*Latimeria chalumnae*), thus indicating a much earlier origin of PP. In comparison, the more ancient genes for both NPY and PYY are present in all major vertebrate groups. It is hypothesised that the initial replication process which led to the separate genes for NPY and PYY took place at the time of the whole genome duplication (in which the multiple HOX gene clusters were produced), suggesting a common ancestral origin. On the other hand, the generation of the PP genes arose from a tandem duplication of the PYY gene at the time of, or immediately before, the emergence of tetrapods (Conlon, 2002; Hort *et al.*, 1995; Larhammar *et al.*, 2004).



**Figure 1.2 The PP-fold adopted by the NPY family.** The PP family is characterised by a polyproline helix, a β-turn, and an interdigitating α-helix as determined by X-ray crystallography of turkey PP (Blundell *et al.*, 1981). Hydrophobic interactions between side chains of the α-helix and the prolines in the N-terminal section ensure that the α-helix and polyproline helix are secured in a folded configuration. The green and grey residues demonstrate alternative hydrophobic polar pairs.

#### 1.1.4 High sequence homology between PP, PYY and NPY

NPY is the most highly conserved between different species, so much so that 33 of the 36 amino acids in *Torpedo marmorata* are identical compared with mammalian NPY. It is thought that the evolution of PYY and NPY involved duplication from a common ancestral gene; the corresponding genes are now located on different chromosomes. Like NPY, PYY is also highly conserved, with an identical amino acid sequence in both

mouse (m) and rat (r) PYY. It is now known that PP arose by duplication of the PYY gene, with both genes situated on chromosome 17q21.1 in close proximity (Hort *et al.*, 1995). In contrast to NPY and PYY, PP exhibits least conservation and thus a high degree of species specificity; for example, mPP and rPP share 94% sequence identity, whereas mPP and hPP share 83% sequence identity (Figure 1.3). A significant loss in sequence identity is observed when comparing mPP to that of chicken (ch), with only 42 % identity between the two species.

#### A: PP

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
hPP	A	P	L	E	P	V	Y	P	G	D	N	A	T	P	E	Q	M	A
mPP	A	P	L	E	P	M	Y	P	G	D	Y	A	T	P	E	Q	M	A
rPP	A	P	L	E	P	M	Y	P	G	D	Y	A	T	H	E	Q	R	A

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
hPP	Q	Y	A	A	D	L	R	R	Y	I	N	M	L	T	R	P	R	Y
mPP	Q	Y	E	T	Q	L	R	R	Y	I	N	T	L	T	R	P	R	Y
rPP	Q	Y	E	T	Q	L	R	R	Y	I	N	T	L	T	R	P	R	Y

#### B: PYY

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
hPYY	Y	P	I	L	P	E	A	P	G	E	D	A	S	P	E	Q	L	N
mPYY	Y	P	A	L	P	E	A	P	G	E	D	A	S	P	E	Q	L	S
rPYY	Y	P	A	L	P	E	A	P	G	E	D	A	S	P	E	Q	L	S

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
hPYY	A	Y	Y	A	S	L	R	H	L	Y	N	L	V	T	R	Q	R	Y
mPYY	A	Y	Y	A	S	L	R	H	L	Y	N	L	V	T	R	Q	R	Y
rPP	A	Y	Y	A	S	L	R	H	L	Y	N	L	V	T	R	Q	R	Y

**Figure 1.3 A comparison of the human, mouse and rat peptide variants of (A) PP and (B) PYY, illustrating sequence homology.** Alterations in the amino acids between the three species are highlighted in blue. Adapted from Larhammar (1996).

#### 1.1.5 Distribution of PP, PYY and NPY

Largely, expression of PP is limited to endocrine cells; PYY is expressed in both endocrine cells and discrete neurone populations, while NPY has a high prevalence in neuronal tissue with some detection additionally in platelets (Ericsson *et al.*, 1987). The

considerable structural similarity between each peptide of the PP family has made the detection of their distribution patterns complex. However, in recent years, the development of new and selective antibodies, coupled with the use of *in situ* hybridisation has enabled the in depth knowledge about the distribution of each member of the NPY family. The major sites of PP, PYY and NPY expression in the periphery, particularly in the GI tract, are outlined below.

#### **1.1.5.1 PP**

PP expression is predominately found in the pancreas; in human pancreas, PP is contained in two distinguishable cell types; the PP-poor dorsal lobe cells and the PP-rich ventral lobe cells. The former cells contain small PP positive electron-dense granules, comparable to those seen in rodents, whereas the latter cells contain PP positive (F-type) cells with much larger and less electron dense granules (Fiocca *et al.*, 1983). Interestingly, cells have been identified in human foetal pancreas as early as 10 weeks using immunohistochemistry, a phenomenon which has not been reported in rodents (Paulin and Dubois, 1978).

In terms of GI expression, very little PP-immunoreactivity has been detected in endocrine cells. In fact, PP immunoreactive cells in the stomach have only been described in opossum, cat and dog (Feurle *et al.*, 1985; Larsson *et al.*, 1976; Sundler *et al.*, 1984), and only seem to correspond to a subpopulation of the gastrin cells, as demonstrated in dog antrum. Only a few PP cells have been detected in the gastric mucosa for a short postnatal period in human (Tsutsumi *et al.*, 1984) and rat (Sundler *et al.*, 1977). Proof of PP immunoreactivity in the intestine is limited; in dog, PP cells seem to be constrained to the duodenum (Sundler *et al.*, 1984; Feurle *et al.*, 1985). Shortly after the discovery of PYY, the detection of PP-containing cells in the distal human bowel was controversial due to the possibility of antibody cross-reactivity (Fiocca *et al.*, 1980; Lehy *et al.*, 1981). Stronger evidence for the presence of PP-immunoreactive enteroendocrine cells in the human colon and rectum (distinguishable from PYY-containing enteroendocrine cells) was subsequently published (el Salhy *et al.*, 1983a; el Salhy *et al.*, 1997). More recently, Habib *et al.* (2012) utilised microarray expression profiling and quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) to demonstrate detectable PP expression in colonic L-cells in the mouse. Interestingly, PP expression was not observed in upper small intestinal L-cells, which is in accordance with the endogenous peptide playing a modulatory peripheral role in



distal colonic ion transport and lower gastrointestinal motility (discussed further in section in 1.5).

#### **1.1.5.2 PYY**

PYY circulates in two active forms: PYY(1-36) and PYY(3-36) (Grandt *et al.*, 1994). The truncated PYY(3-36) results from the cleavage of the tyrosine-proline residues from the N-terminal end of the polypeptide via DPPIV (Medeiros and Turner, 1994). Grandt and colleagues found that PYY(1-36) accounted for 60% of the PYY immunoreactivity in fresh canine colon and the truncated form, PYY(3-36), represented the remaining 40% of immunoreactivity (Grandt *et al.*, 1994).

The major source of PYY in the GI tract is the endocrine L-cell (Ekblad and Sundler, 2002; Ueno *et al.*, 2008), which increase in density along the proximal–distal gut axis; hence the largest source of PYY is found in the distal gut. PYY-positive L-cells contain the proglucagon-derived peptide, glicentin (Böttcher *et al.*, 1984; Solcia *et al.*, 1985), glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) (Ekblad and Sundler, 2002). Early studies performed in human tissue by Sjölund *et al.* revealed ~45% of PYY- immunoreactive cells are found in the rectum, ~27% in the colon and ~4% in the jejunum. These expression levels were based on the number of glicentin immunoreactive cells in the lower gut (Sjölund *et al.*, 1983). More recently, Habib *et al.* reported a high proportion of L-cells in the upper small intestine which expressed message for a range of gut hormones, including gastric inhibitory polypeptide (GIP), cholecystokinin (CCK), secretin, neurotensin and PYY, with the latter being expressed in 20 % of all upper small intestinal L-cells. Furthermore, Habib *et al.*, corroborated earlier studies by revealing that a large number of colonic L-cells co-expressed GLP-1 and PYY, and that the percentage of L-cells containing PYY increased progressively towards the colon, demonstrated through immunostaining and fluorescence-activated cell sorting analysis (Habib *et al.*, 2012).

Besides its intestinal endocrine cell expression, evidence has been provided for the localisation of PYY in specific enteric neurones. Böttcher *et al.* demonstrated the existence of PYY immunoreactivity in the upper GI tract of the rat, cat, ferret and pig by immunocytochemistry and radioimmunoassay combined with high performance liquid chromatography. In the rat, PYY-immunoreactive nerve fibres were virtually restricted to the stomach smooth muscle of the minor curvature where its expression was

abundant. In the cat and ferret, PYY-immunoreactive nerve fibres occurred in the circular smooth muscle layer of both the minor and major curvatures of the stomach and also in the upper small intestine; these fibres were also abundant in myenteric ganglia in these regions. In 1983, El Salhy *et al.* demonstrated that the enteric nerves containing PYY were distinct from those containing NPY (El Salhy, 1983), and this evidence was further supported by Bottcher *et al.* who identified PYY in extracts of enteric ganglia with a radioimmunoassay not recognising NPY (Bottcher *et al.*, 1984).

#### **1.1.5.3 NPY**

Contrary to the endocrine localisation of the PP and PYY, peripheral NPY is widely expressed in the enteric nervous system (ENS), as well as in extrinsic sympathetic and parasympathetic neurones (Furness, 2006). Within the gut, the ENS constitutes both myenteric and submucosal ganglia which integrate with the muscular and mucosal regions (see section 1.5 for details). Studies with the guinea pig small intestine have revealed that NPY is localised in three distinct neurone types: myenteric inhibitory motor neurones that innervate circular smooth muscle; descending myenteric interneurones; and submucosal secretomotor (nonvasodilator) neurones (Sang and Young, 1996). Examination of mouse small intestine has also revealed that 26 % of the total numbers of neurones of the myenteric plexus express NPY, with expression in cell bodies and nerve fibres (Sang and Young, 1996). Interestingly, co-localisation studies in the myenteric cell bodies have demonstrated that NPY is co-localised with vasoactive intestinal polypeptide (VIP) in 50% of all VIP positive neurones. In addition, co-localisation of NPY with nitric oxide synthase (NOS) has been demonstrated in 66% of all NPY positive myenteric cell bodies. In the human colon, the submucosal plexus comprises of three distinguishable layers of ganglia; Henle's plexus, which is found close to the circular muscle, Meissner's plexus, which is found next to the muscularis mucosa; and an intermediate plexus located between Henle's plexus and Meissner's plexus, and in close proximity to the blood vessels of the submucosa (Hoyle and Burnstock, 1989). Evidence from Hoyle and Burnstock did initially contradict another early study which demonstrated little NPY immunoreactivity in adult sigmoid colon (Crowe *et al.*, 1992); however Nichols *et al* (1994) demonstrated its presence in 41% of neuronal cell bodies identified in all three ganglia of the infant submucosal plexus. As a result, this group hypothesised a potential role for NPY in epithelial function due to discovery of extensive innervations of varicose fibres to the mucosa.

## 1.2 The Y receptor family

### 1.2.1 The categorisation and cloning of different Y receptor types

Historically, the NPY receptors were categorised into the different types following the observation that C-terminal fragments of NPY or PYY, e.g. NPY(13–36) could mimic various responses to NPY such as pre-junctional inhibition of twitch responses in the rat vas deferens, but were unable to cause vasoconstriction in guinea pig iliac vein (Wahlestedt *et al.*, 1986). From these findings, the Y<sub>1</sub> and Y<sub>2</sub> receptor types were distinguished based on their ability to become activated by either full length peptide sequences or the C-terminal fragments, respectively. With the use of PYY and NPY analogues with Pro substitutions at residue 34 (as is the case with PP), the categorisation of the receptors were further supported, as the two peptides exhibited increased potency at the Y<sub>1</sub> receptor and reduced efficacy at Y<sub>2</sub> receptor following the amino acid substitution (Fuhlendorff *et al.*, 1990; Grandt *et al.*, 1994). Subsequently, additional receptor types came to light; in 1986, Gilbert *et al.* injected radiolabelled bovine (b) PP into dogs and binding in small intestinal mucosa was analysed (Gilbert *et al.*, 1986). For the first time, this group reported receptors to have preference for PP over PYY and NPY. Two years later, the same group revealed PP-preferring receptors in displacement binding studies using [<sup>125</sup>I] PP in isolated preparations of canine intestinal mucosa (Gilbert *et al.*, 1988). In 1991, PP binding to the canine intestinal receptor and receptor cross-reactivities to various PPs and bPP fragments led to the discovery that PP required an intact N-terminus, since loss of the first two amino acids resulted in the affinity being reduced to half (Gingerich *et al.*, 1991). The same group went on to demonstrate that not only were the first 4 amino acids of the PP sequence essential for binding to the receptor, but also that intact amidated C-terminal tyrosine was necessary for full agonism with the peptide.

With an NPY preferring phenotype, the “Y<sub>3</sub>” receptor was described in rat colon (Dumont *et al.*, 1994), although no receptor has been cloned, and for this reason it is no longer thought to exist. Before the existence of selective Y receptor antagonists, a novel fourth receptor was hypothesised in rabbit colon, since PP, PYY and NPY were able to inhibit anion secretion (Ballantyne *et al.*, 1993); however, using mouse and human colon, the reduction in ion secretion was demonstrated to be the result of stimulation of multiple receptor types; Y<sub>1</sub>, Y<sub>2</sub> and Y<sub>4</sub> receptors (Cox *et al.*, 2001a; Cox and Tough, 2002).

Progress in molecular biology techniques resulted in the identification of five NPY receptor types, Y<sub>1</sub>, Y<sub>2</sub>, Y<sub>4</sub>, Y<sub>5</sub> and y<sub>6</sub>. All NPY receptors are seven-transmembrane (TM) domain G protein-coupled receptors (GPCRs), which couple preferentially to the G<sub>i</sub>/G<sub>o</sub> family, and are thus sensitive to pertussis toxin. The receptor types share only 30-50% sequence identity and are characterised by distinct tissue localisations and unique pharmacological profiles (discussed in more detail below). Based on this, each type is placed into one of three groups; the Y<sub>1</sub>-like receptors, the Y<sub>2</sub>-like receptors, and the Y<sub>5</sub>-like receptors. The Y<sub>1</sub>-like receptors consist of the Y<sub>1</sub>, Y<sub>4</sub> and y<sub>6</sub> receptors, the Y<sub>2</sub>-like receptors include the Y<sub>2</sub> receptor and the Y<sub>7</sub> receptor which was more recently isolated from zebrafish and frog (Fredriksson *et al.*, 2004) and the Y<sub>5</sub>-like receptors which consists of the Y<sub>5</sub> receptor only. In 2004, Larhammar *et al.* demonstrated that Y<sub>5</sub> receptor is more phylogenetically similar to the Y<sub>1</sub> subfamily compared to the Y<sub>2</sub>-like receptors. Table 1.1 indicates the agonists and antagonists associated with each mammalian Y receptor type and their relative potencies.

	Y <sub>1</sub>	Y <sub>2</sub>	Y <sub>4</sub>	Y <sub>5</sub>	y <sub>6</sub>
G-protein	G <sub>i/o</sub> , ↓cAMP	G <sub>i/o</sub> , ↓cAMP	G <sub>i/o</sub> , ↓cAMP	G <sub>i/o</sub> , ↓cAMP	G <sub>i/o</sub> , ↓cAMP
Order of potency	PYY ≈ NPY > PYY(3-36) >>PP	PYY ≈ NPY ≈ PYY(3-36) >>PP	PP >> PYY > NPY > PYY(3-36)	NPY ≈ PYY ≈ PP > PYY(3-36)	PYY ≈ NPY > NPY(3-36) >>PP
Selective agonists	Pro <sup>34</sup> NPY, Pro <sup>34</sup> PYY	NPY(3-36) PYY(3-36)	PP, GR231118	[Ala <sup>31</sup> , Aib <sup>32</sup> ]pNPY	-
Antagonist	BIBP3226 BIBO3304 GR231118	BIIE0246	-	CGP71683A	-

**Table 1.1 The pharmacology corresponding to each mammalian Y receptor types.**

### 1.2.1.1 The Y<sub>1</sub> receptor

The Y<sub>1</sub> receptor was the first of the NPY receptor family to be identified; cDNA was cloned from rat forebrain (Eva *et al.*, 1990) which was subsequently found to encode the Y<sub>1</sub> receptor (Krause *et al.*, 1992; Petitto *et al.*, 1994; Herzog *et al.*, 1992).

Thereafter, homologs from various species such as humans (Larhammar *et al.*, 1992), mice (Eva *et al.*, 1992) and *Xenopus laevis* (Blomqvist *et al.*, 1995) were published. 384 amino acid residues encompass the hY<sub>1</sub> receptor and display a 94% sequence homology identity with the rY<sub>1</sub> and mY<sub>1</sub> receptors. Y<sub>1</sub> transfection studies have revealed an order of potency of full length NPY and PYY ≥ Pro<sup>34</sup> analogues >> C-terminal fragments >

PP (Larhammar *et al.*, 1992; Eva *et al.*, 1992; Krause *et al.*, 1992), as indicated by early pharmacological studies. BIBP3226, a non-peptide antagonist developed in 1995, exhibits high potency for the Y<sub>1</sub> receptor (with K<sub>i</sub> or K<sub>B</sub> values of between 1 to 10 nM) and importantly, its affinity at the Y<sub>2</sub>, Y<sub>4</sub> and Y<sub>5</sub> receptor exceeds 10 µM (Wieland *et al.*, 1995; Gerald *et al.*, 1996). A further selective antagonist at the Y<sub>1</sub> receptor, BIBO3304, was developed shortly afterwards; this antagonist displayed a 1000-10000-fold lower affinity for the human and rat Y<sub>2</sub>, Y<sub>4</sub> and Y<sub>5</sub> receptor, compared to subnanomolar affinity at the Y<sub>1</sub> receptor in SK-N-MC cells (Wieland *et al.*, 1998). Additional Y<sub>1</sub> antagonists have been reported, including SR120819A or GR231118, however little data exists regarding their selectivity towards the Y<sub>5</sub> and y<sub>6</sub> receptors (Daniels *et al.*, 1995; Serradeil- Le Gal *et al.*, 1995).

### 1.2.1.2 The Y<sub>2</sub> receptor

Y<sub>2</sub> receptor cDNA was first cloned from human SMS-KAN cells (Rose *et al.*, 1995) and thereafter from human brain cDNA libraries (Gehlert *et al.*, 1996; Gerald *et al.*, 1995) and the human neuroblastoma cell line KAN-TX (Rimland *et al.*, 1996). In later studies, the receptor was cloned from mouse (Nakamura *et al.*, 1996) and rat brain (St Pierre *et al.*, 1998). In terms of Y<sub>2</sub> receptor pharmacology, the receptor exhibits an order of potency of NPY  $\approx$  PYY  $\geq$  C-terminal fragments  $\gg$  Pro<sup>34</sup> analogues  $>$  PP. The hY<sub>2</sub> receptor is 381 amino acids long and shares 92% and 94% identity with the rY<sub>2</sub> and mY<sub>2</sub> variants, respectively. Currently, the non-peptide antagonist BIIE0246 exhibits the highest potency selective for the Y<sub>2</sub> receptor (Doods *et al.*, 1999), and is extensively used to study this receptor type.

### 1.2.1.3 The Y<sub>4</sub> receptor

The Y<sub>4</sub> receptor gene was at first cloned from a human genomic library and named “PP1” (Lundell *et al.*, 1995). Since then, Y<sub>4</sub> receptors from several other species have been isolated and characterised including the murine receptors (Gregor *et al.*, 1996a) and the rat (Lundell *et al.*, 1996; Yan *et al.*, 1996). The Y<sub>4</sub> receptor is comprised of 375 amino acids, with 75% of the sequence being identical to the rY<sub>4</sub> and 76% sequence being shared with the mY<sub>4</sub>. Between the rY<sub>4</sub> and mY<sub>4</sub>, a 94% identity exists between the two sequences. Compared with the other members of the NPY family, PP exhibits the most preference for the Y<sub>4</sub> receptor, followed by Pro<sup>34</sup>PYY  $>$  PYY  $\geq$  Pro<sup>34</sup>NPY  $>$  NPY  $>$  PYY C-terminal fragments  $>$  NPY C-terminal fragments  $>$  hPP C-terminal fragments (Bard *et al.*, 1995), determined by studies on canine intestinal mucosa. The same group

also reported a high extent of ligand-receptor specificity between different species since a variation in potency for the Y<sub>4</sub> receptor was observed; hPP  $\approx$  bPP > rPP > frog PP. This species specificity appears to be unique to the Y<sub>4</sub> receptor compared with any other member of the NPY receptor family. To date, a high affinity Y<sub>4</sub> receptor antagonist has not yet been developed, however GR231118, a dipeptide founded from the C-terminal sequence of NPY (Daniels *et al.*, 1995), has been reported to be a partial agonist at the Y<sub>4</sub> receptor (Tough *et al.*, 2006), exhibiting picomolar affinity (Gehlert *et al.*, 1996).

#### 1.2.1.4 The Y<sub>5</sub> receptor

In terms of sequence homology, the hY<sub>5</sub> and rY<sub>5</sub> receptors are 88% identical and are both comprised of 445 amino acids. Interestingly, the mY<sub>5</sub> receptor consists of 466 amino acids and has a 97% sequence identity to the rY<sub>5</sub> and 89% with its human equivalent. The Y<sub>5</sub> receptor was first cloned in 1996 from the rat hypothalamus (Gerald *et al.*, 1996), followed by cloning from mouse (Weinberg *et al.*, 1996; Nakamura *et al.*, 1997) and human (Hu *et al.*, 1996) genomic libraries, in addition to a number of other species. Initial studies utilising rY<sub>5</sub> receptor-transfected LM-TK-cells suggested that NPY, PYY, Pro<sup>34</sup> analogues, small C-terminal fragments and hPP were all capable of activating the Y<sub>5</sub> receptor with equal potency (Gerald *et al.*, 1996). Subsequent displacement studies however, indicated a much lower affinity with rPP compared with its human counterpart, when using transfected COS-7 cells and [<sup>125</sup>I]PYY (Borowsky *et al.*, 1998). Given its extensive central localisation and principal role in food intake, much attention has been paid to the Y<sub>5</sub> receptor, and several groups have attempted to develop selective agonists and antagonists to further elucidate its significance. Initial studies successfully utilised the Y<sub>5</sub> agonist, [D-Trp<sup>32</sup>]NPY (Gerald *et al.*, 1996), although later it was reported that peptides possessing Ala<sup>31</sup>, Aib<sup>32</sup>, the recognition motif for the Y<sub>5</sub> receptor, are more favourable at this receptor type (Cabrele *et al.*, 2000; Cabrele *et al.*, 2002; Dumont *et al.*, 2003; Hoflinger *et al.*, 2003). In 1998, the first non-peptidic potent antagonist at the Y<sub>5</sub> receptor was reported by Criscione and co-workers (Criscione *et al.*, 1998); CGP71683A inhibited the effects of NPY on intracellular Ca<sup>2+</sup> levels in a concentration-dependent manner *in vitro*. The same group also demonstrated complete inhibition of the effects of NPY with concentrations of the Y<sub>5</sub> antagonists as low as 50 nM (Criscione *et al.*, 1998).

### 1.2.1.5 The $y_6$ receptor

To date, the  $y_6$  receptor has been isolated from dogfish (Salaneck *et al.*, 2003) guinea pig (Starback *et al.*, 2000), mouse (Gregor, *et al.* 1996b), pig (Wraith *et al.*, 2000) and chicken (Bromée *et al.*, 2006) but interestingly the receptor appears to be completely absent from the rat (Burkhoff *et al.*, 1998). In human, guinea pig and pig, the  $y_6$  receptors exist as pseudogenes resulting in non-functional proteins, in contrast to the mouse and rabbit, in which the  $y_6$  receptor is functional. The existence of the  $y_6$  pseudogenes in humans is thought to occur as a result of a frameshift mutation; a single base deletion in the coding sequence of the predicted third intracellular loop results in a stop codon in TM6 (Gregor *et al.*, 1996a). Contradictory radioligand binding data exists regarding the pharmacological profile of the murine  $y_6$  receptor; in 1996, Weinberg *et al.* reported an order of potency of  $\text{NPY} \approx \text{PYY} \approx [\text{Pro}^{34}]$  substituted analogue  $> \text{NPY}(13-36) \gg \text{PP} (>1000\text{nM})$  (Weinberg *et al.*, 1996), however in the same year Gregor *et al.* reported an order of potency of  $\text{PP} \gg [\text{Pro}^{34}]$  substituted analogue  $\gg \text{PYY} \approx \text{NPY} > \text{C-terminal fragment (NPY}(3-36), \text{NPY}(13-36), \text{each} > 1000 \text{ nM})$  (Gregor *et al.*, 1996b).

### 1.2.2 Distribution of Y receptors in the GI tract

The  $Y_1$  receptor has an widespread distribution pattern and extensive use of Northern analysis, *in situ* hybridisation and RT-PCR has now confirmed expression in both the central nervous system (CNS) and various peripheral organs such as the heart, skeletal muscle, lung, adrenal glands and intestinal tract (Wharton *et al.*, 1993; Ball *et al.*, 1995; Nakamura *et al.*, 1995; Goumain *et al.*, 1998).  $Y_1$  receptor immunoreactivity in the intestinal tract has been reported in human (Peaire *et al.*, 1997; Mannon *et al.*, 1999), rat colon (Jackerott and Larsson, 1997) and mouse and rat small intestine (Matsuda *et al.*, 2002). The  $Y_1$  receptor has an extensive distribution pattern in both myenteric and submucosal neurones in the human colon, detected by positive immunoreactivity (Peaire *et al.*, 1997). This group highlighted staining in fibres along the longitudinal and circular smooth muscle and the muscularis mucosa (Peaire *et al.*, 1997).  $Y_1$  labelling is also co-localised with NPY positive cell soma in Henle's plexus of human colon. This provides evidence of a pre-synaptic inhibitory role for the  $Y_1$  receptor on NPY release. This NPY and  $Y_1$  co-localisation however is not seen in the myenteric plexus but instead, NPY positive nerve fibres are surrounded by  $Y_1$  positive cell bodies (Peaire *et al.*, 1997).  $Y_1$  receptor expression is also present the basolateral domain of epithelial cells, in close proximity to PYY positive endocrine L-cells. This indicates that PYY

released from L-cells into the lamina propria has the potential to exert significant paracrine, epithelial-derived Y<sub>1</sub> actions in addition to modulating neuronal mechanisms (Mannon *et al.*, 1999).

Similarly to the Y<sub>1</sub> receptor, Y<sub>2</sub> receptor expression is found in both central and peripheral locations. *In situ* hybridisation and Northern analysis has demonstrated the expression of central Y<sub>2</sub> gene transcripts in the rat (Zhang *et al.*, 1997; Gustafson *et al.*, 1997; St Pierre *et al.*, 1998), mouse (Diez *et al.*, 1997; Naveilhan *et al.*, 1998) and man (Rose *et al.*, 1995; Gehlert *et al.*, 1996; Caberlotto *et al.*, 2000). Unlike the Y<sub>1</sub> receptor, the distribution patterns of Y<sub>2</sub> receptors in the mammalian ENS are more discrete, and are based on information from RT-PCR, autoradiographic and functional studies. The limited availability of selective Y<sub>2</sub> receptor antibodies has led to only a small number of published immunohistochemical studies. Ferrier *et al.* examined Y<sub>2</sub> mRNA levels in human intestinal segments and reported high levels in muscle layers of the ileum and left colon and in mucosal layers of the ileum and right colon, however the same group were unable to detect Y<sub>2</sub> receptor mRNA in muscle preparations of rat intestine (Ferrier *et al.*, 2002). Despite Y<sub>2</sub> receptor-mediated NPY contractions being observed in rat proximal colon (Pheng *et al.*, 1999), there was no Y<sub>2</sub> receptor mRNA detected in nerve-muscle preparations of the same species (Ferrier *et al.*, 2000). These studies collectively provide evidence that Y<sub>2</sub> receptors are predominantly neuronal. Y<sub>2</sub> expression on submucous and myenteric plexi indicates a neuromodulatory role in the ENS. This idea was further supported by Hyland *et al.* (2003) who demonstrated pre-junctional localisation of Y<sub>2</sub> receptors in isolated mouse mucosa and showed a reduction in epithelial ion transport via PYY(3-36). More recently, Wang *et al.* (2010) detected Y<sub>2</sub> receptor transcripts using RT-PCR, both in the mouse mucosa layer and submucosa plus muscle layers. This group detected high levels of receptor expression in the mucosa compared to other layers of the proximal and distal colon, and comparison of the two colonic preparations revealed a higher signal in the distal than proximal colon. With the use of a polyclonal rabbit anti-Y<sub>2</sub> serum, immunofluorescent staining revealed Y<sub>2</sub> localisation in the mouse submucosal and myenteric plexi. As with internal controls, pre-absorption of the primary antibody with the Y<sub>2</sub> immunogenic peptide (TDSFSEATN-COOH) was performed to establish antibody specificity. As a result, Y<sub>2</sub> labelling was established mainly in nerve varicosities around neurone cell bodies and running between ganglia.



*In situ* hybridisation has allowed the identification of Y<sub>4</sub> receptor gene transcripts in rat tissue. In human peripheral tissue, Y<sub>4</sub> receptor mRNA has been detected in the intestinal tract, the heart and reproductive organs, with lower levels being reported in the pancreas and kidney (Lundell *et al.*, 1995; Bard *et al.*, 1995; Yan *et al.*, 1996; Gregor *et al.*, 1996b). Several studies have focused on Y<sub>4</sub> receptor expression in the human gastrointestinal tract, and it has now been established that Y<sub>4</sub> mRNA is present in the small and large bowel (Lundell *et al.*, 1995), as determined by northern analyses. Constitutive Y<sub>4</sub> receptor expression has been detected in three human colonic adenocarcinoma cell lines with the use of RT-PCR (Cox *et al.*, 2001b). When pre-treated with VIP, these cell lines exhibit consistent inhibitory responses to low nM concentrations of hPP (Cox & Tough, 1995; Holliday *et al.*, 1997; Cox *et al.*, 2001b), an effect that is also observed in human colon mucosa and mouse descending colon mucosa (Tough *et al.*, 2006). One particular study demonstrated Y<sub>4</sub> receptor localisation on rat goblet cells and on intestinal villi, specifically on the basal lamina (Campbell *et al.*, 2003), suggesting a number of potential undefined roles for the Y<sub>4</sub> receptor, such as mucus secretion in the intestine.

Contrary to the extensive peripheral and central localisation patterns for the Y<sub>1</sub>, Y<sub>2</sub> and Y<sub>4</sub> receptor, the Y<sub>5</sub> receptor has very limited expression in peripheral tissue and is largely localised to the CNS. In rat GI tissue, Y<sub>5</sub> mRNA was not measured in the fundus, antrum, duodenum, jejunum, ileum, caecum or distal colon (Ferrier *et al.*, 2002). The same group also reported lack of Y<sub>5</sub> mRNA human ileum, colon, and rectal tissues. Y<sub>5</sub> mRNA is however present at low levels, in rat proximal colon (Feletou *et al.*, 1998) in colonic muscle layers and possibly in jejunal crypts (Goumain *et al.*, 1998) although functional data indicates that this receptor type has no functional role in rodent or human intestinal ion transport responses; Cox *et al.* (2001a) studied the effects of the Y<sub>5</sub> agonist, Ala<sup>31</sup>, Aib<sup>32</sup>hNPY on ion secretion in mouse isolated colon mucosa and demonstrated that Ala<sup>31</sup>, Aib<sup>32</sup>hNPY was only active at micromolar concentrations. This suggests that Y<sub>5</sub> receptors do not play a significant role in reducing ion secretion.

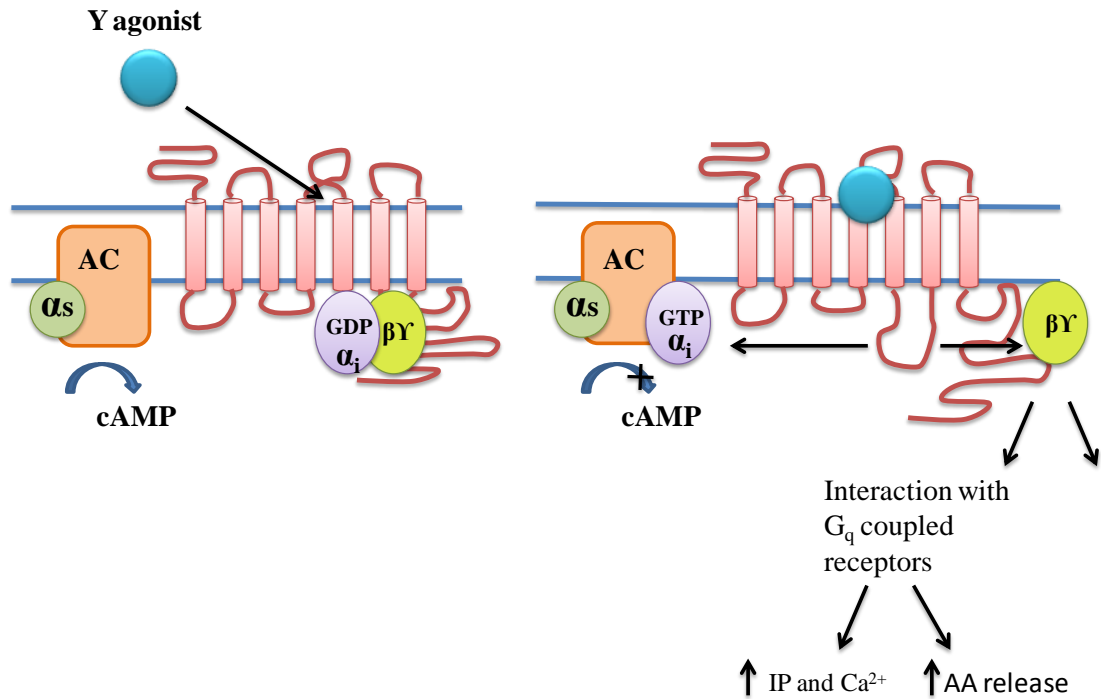
The y<sub>6</sub> receptor is a pseudogene in primates, containing a single base pair deletion in the sixth transmembrane domain resulting in a truncated, non-functional receptor (Matsumoto *et al.*, 1996). Peripheral expression of the y<sub>6</sub> receptor is limited; it is not expressed in rat but is expressed in the mouse; y<sub>6</sub> mRNA has been detected in the small intestine (and at lower levels in embryonic colon, Gregor *et al.*, 1996a, although the

detailed distribution patterns have not been described. Messenger RNA for the  $y_6$  receptor has also been identified in human and rabbit small intestine and colon (Gregor *et al.*, 1996a; Matsumoto *et al.*, 1996) however, the lack of  $y_6$  receptor antibody has hindered the detection of protein expression.

Taken together, the patterns of peptide and receptor localisation in the intestine show variation along the length of the gut within species, as well as clear differences between species. Given their extensive presence within the basolateral aspect of the epithelial layer ( $Y_1$  and  $Y_4$  receptors) or on enteric neurones ( $Y_1$  and  $Y_2$  receptors) and their activation by endogenous NPY, PYY ( $Y_1$  and  $Y_2$  receptors) or PP (which prefers  $Y_4$  receptors), it is evident that these receptors play a significant functional role in the human GI tract, as well as in particular rodent models (e.g. mouse).

### **1.2.3 Signalling and internalisation of the Y receptors, with a focus on $Y_1$ , $Y_2$ and $Y_4$ receptors and their desensitisation**

Each Y receptor signals via pertussis toxin-sensitive G proteins, e.g. members of the  $G_i$  and  $G_o$  family, which mediate the inhibition of adenylyl cyclases (AC), and consequently, the inhibition of cyclic adenosine monophosphate (cAMP) synthesis (Figure 1.4). In their inactive form G proteins are found in a heterotrimeric complex with guanosine-5'-diphosphate (GDP) bound to the  $\alpha$  subunit ( $\alpha$ GDP $\beta\gamma$ ). Following agonist-mediated receptor activation, a conformational change in the  $G_\alpha$  subunit promotes exchange of GDP for guanosine-5'-triphosphate (GTP). GTP binding results in the complex dissociating into a  $\beta\gamma$  dimer and the free, active  $\alpha$ GDP subunit. The  $G_\alpha$  subunit contains an integral GTP-ase and once AC is inhibited, the  $G_\alpha$  subunit hydrolyses GTP to GDP and re-associates with the  $\beta\gamma$  subunit.

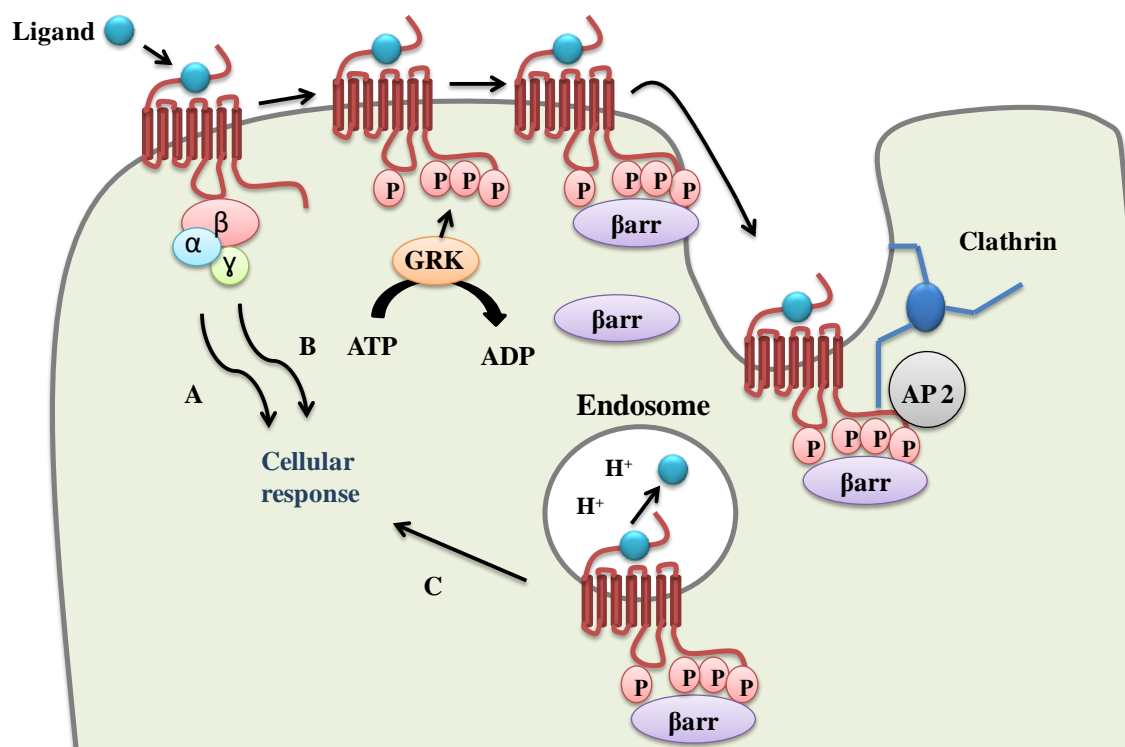


**Figure 1.4 Signal transduction following Y receptor activation.** Preceding agonist binding,  $G_\alpha$  and  $G_{\beta\gamma}$  subunits exist in a heterotrimeric complex and interact with the Y receptor C-terminus. Adenylate cyclase, stimulated by the  $G_s$  subunit from another receptor, stimulates cAMP production. Following activation, the  $G_\alpha$  and  $G_{\beta\gamma}$  subunits dissociate and the active, unrestrained  $G_\alpha$  inhibits cAMP production whereas the  $G_{\beta\gamma}$  subunit can interact with  $G_q$  coupled receptors to stimulate inositol phosphate (IP) production, elevate intracellular  $Ca^{2+}$  levels, increase arachidonic acid (AA) release and activate mitogen activated protein kinases (MAPK). Adapted from Holliday *et al.* (2005) and Tough (2006).

The onset of GPCR desensitisation is a mechanism in which there is a loss in intracellular signalling, despite the presence of a ligand (Ferguson, 2001). This process is the consequence of a combination of different mechanisms. The cloning of the  $\beta_2$ -adrenergic receptors ( $\beta_2$ -AR) in 1986 and its shared homology with the rhodopsin receptor (Sibley *et al.*, 1986) led to the understanding of receptor desensitisation. Now, GPCRs are traditionally thought to signal via activation of heterotrimeric G proteins and then desensitise by G protein-coupled receptor kinases (GRKs) (Ribas *et al.*, 2007), which are recruited to and specifically phosphorylate agonist-occupied receptors leading to “homologous desensitisation” (Benovic *et al.*, 1986; Hausdorff *et al.*, 1990). GRKs phosphorylate GPCRs at both serine and threonine residues localised within the third intracellular loop or C-terminal tail domain (Premont *et al.*, 1995). GRK-phosphorylation of receptors is not sufficient for desensitisation, but instead serves to create high affinity sites to promote the recruitment of the cytoplasmic accessory proteins, arrestins, and target the receptors for internalisation via clathrin-coated pits. The arrestin protein was first identified by Wilden *et al.* in 1986 as a 48K protein,

bound to the phosphorylated rhodopsin, thereby interfering with its coupling to transducin (Wilden *et al.*, 1986). The protein was later renamed arrestin (Zuckerman and Cheasty, 1986). The arrestin recruitment subsequently induces desensitisation by preventing further coupling to G proteins (Lohse *et al.*, 1990).

Besides their role in desensitisation,  $\beta$ -arrestins recruit GPCRs such as the  $Y_1$  receptor to clathrin coated pits and as a consequence, the receptor undergoes rapid internalisation (Gicquiaux *et al.*, 2002; Parker *et al.*, 2002; Pheng *et al.*, 2003; Holliday *et al.*, 2005). This process is thought to occur through interactions with the endocytotic machinery, such as the clathrin heavy chain or the clathrin adaptor protein AP2 (Goodman *et al.*, 1996; Laporte *et al.*, 1999) (Figure 1.5). Dynamin then facilitates the detachment of clathrin-coated pits from the plasma membrane (Doherty & McMahon, 2009). Desensitisation is an essential process which is required to prevent over-activation in response to persistent ligand stimulation. In the same way, to avoid prolonged desensitisation, GPCRs have the capacity to reactivate or resensitise once the ligand has been removed. Once internalised, GPCRs are either: rapidly targeted to the lysosome for its degradation resulting in complete termination of receptor signal activity, rapidly recycled back to the plasma membrane resulting in resensitisation and hormone signal recovery, or are retained in endosomes, crossing over to the degradative or recycling pathways at a much slower rate (Hanyaloglu and Zastrow, 2008; Marchese *et al.*, 2008).



**Figure 1.5 Schematic diagram of signal transduction in GPCRs following receptor activation.** Arrows A and B illustrate ligand-activated receptors which signal to downstream effects via  $\alpha$  and/or  $\beta\gamma$  complexes. Subsequent to phosphorylation of the receptor, arrestin is recruited from the cytoplasmic domain, promoting receptor endocytosis via clathrin-coated pits. Recent published data suggests that a number of GPCR-mediated signalling events may possibly occur from an endosomal compartment (arrow C). Adapted from Hanyaloglu and Zastrow, 2008.

Rapid  $Y_1$  and  $Y_4$  receptor desensitisation has been well documented with the use of cAMP assays in human embryonic kidney (HEK) 293 cells (Gicquiaux *et al.*, 2002) and also in ion transport studies using epithelia (Cox and Tough, 1995; Cox *et al.*, 2001b; Holliday and Cox, 2003; Holliday *et al.*, 2005). Early reports by one group suggested that h $Y_4$  receptor was not susceptible to agonist-induced desensitisation or internalisation in Chinese hamster ovary (CHO) cells (Voisin *et al.*, 2000). One explanation for this may be the long-term (24 hours) PP pre-treatment used in that study resulting in the inability to measure degradation of the agonist. The same group also reported the inability of C-terminal tagged h $Y_4$  receptors to redistribute from the plasma membrane after 24 hour pre-treatment with hPP, however this was contradictory to findings reported by Parker *et al.* (2001 & 2002) whereby it was shown that [ $^{125}$ I]PP was effectively and rapidly sequestered from the plasma membrane following binding to rat  $Y_4$  receptors in the same cell line. This rapid  $Y_4$  desensitisation was later found to be highly associated with  $\beta$ -arrestin-3, verified by bioluminescence resonance energy transfer (BRET) studies (Berglund *et al.*, 2003a). A comparison of the recovery times

for each Y receptor has shown that the Y<sub>4</sub> receptor is much slower than the Y<sub>1</sub> receptor (Parker *et al.*, 2001), but has faster kinetics compared with the Y<sub>5</sub> receptor (Parker *et al.*, 2003; Bohme *et al.*, 2008).

In contrast to the rapid internalisation seen with the Y<sub>1</sub> and Y<sub>4</sub> receptors, the Y<sub>2</sub> receptor interestingly has been reported to internalise slowly (Berglund *et al.*, 2003a; Gicquiaux *et al.*, 2002; Ouedraogo *et al.*, 2008). This slow internalisation is thought to be attributed to slower interactions with arrestin. Berglund *et al.* provided further evidence to support this hypothesis and showed that the Y<sub>1</sub> receptor rapidly recruits the adaptor protein whereas Y<sub>2</sub> receptor recruitment of  $\beta$ -arrestin is much slower (Berglund *et al.*, 2003b) or not observed (Ouedraogo *et al.*, 2008). Using BRET techniques, this group also demonstrated that differences in radiolabelled agonist internalisation of the other Y receptors i.e. Y<sub>1</sub>, Y<sub>4</sub>, Y<sub>5</sub> > Y<sub>2</sub> also broadly corresponds to their affinity for  $\beta$ -arrestin 2 (i.e. Y<sub>1</sub>, Y<sub>5</sub> > Y<sub>4</sub> > Y<sub>2</sub>) (Berglund *et al.*, 2003b). More recently, NPY was shown to have a significantly lower potency at the Y<sub>2</sub> receptor in terms of arrestin stimulation compared to the Y<sub>1</sub> receptor in a bimolecular fluorescence complementation assay (Kilpatrick *et al.*, 2010), however this data did not corroborate earlier fluorescence microscopy studies, whereby it was shown that the Y<sub>2</sub> receptor has a similar rate of internalisation to the Y<sub>1</sub> receptor (Bohme *et al.*, 2008; Lindner *et al.*, 2009).

A vast amount of published data has suggested a key role for the Y<sub>2</sub> and Y<sub>4</sub> receptor in the anorexigenic response to feeding (see section 1.3 for details). From a clinical perspective, the evidence described above for the Y<sub>2</sub> receptor suggests that its low association with arrestin may potentially reduce the risk of developing tolerance, a current problem observed with anti-obesity CCK analogues (Moran and Dailey, 2009). The differential endocytic pathways seen with the Y<sub>2</sub> and Y<sub>4</sub> receptors calls for the need to investigate the clinical repercussions if these receptors are to be targeted in treatment of obesity and to ascertain whether these processes can be controlled to accomplish greater therapeutic potential.

### **1.3 The role of the PP family in satiety**

The accumulation of body fat to a degree which is disadvantageous to health has become a major concern in the western world. Regardless of its pandemic proportions, high morbidity and mortality rates and the financial impact on governments, obesity still

remains a severe health problem in both children and adults. The onset of obesity not only increases the risk of developing serious chronic conditions such as type 2-diabetes, hypertension and coronary heart disease, but is also associated with several types of cancer (Neary *et al.*, 2003; Meister, 2007).

### **1.3.1 Current treatments for obesity**

Currently, the only successful treatment for achieving significant weight loss is surgery. Despite improving morbidity and mortality rates in obese patients (Sjostrom, 2008), these procedures are associated with a peri-operative risk of mortality of 0.5% (Mark *et al.*, 2008). Pharmacological treatments including the drugs orlistat, sibutramine and rimonabant have not been as successful as initially predicted, due to their limited efficacy and unwanted adverse effects; the centrally acting 5-HT/NA (5-hydroxytryptamine/noradrenaline) reuptake inhibitor, Sibutramine, was recently removed from the European market, as evidence suggested an increase in cardiovascular events including hypertension, tachycardia, arrhythmias, myocardial infarction (Sharma *et al.*, 2009). Likewise, the cannabinoid (CB) receptor antagonist, Rimonabant, was also recalled from the market as a result of high incidences in anxiety and depression (Christensen *et al.*, 2007). The peripherally acting GI lipase inhibitor, Orlistat is currently the only pharmacotherapy on the European market for use in obese patients; however, its use is associated with unwanted side effects include intestinal flatulence, borborygmi (a rumbling noise made by the movement of fluid and gas in the intestines), abdominal cramps and faecal incontinence (Rucker *et al.*, 2007), thus resulting in poor tolerability and minimal weight loss. A few currently marketed drugs, approved for other indications, can also be useful for the treatment of obesity. Since obesity is commonly associated with insulin-resistance and type 2 diabetes, currently available drugs are GLP-1 receptor agonists (exenatide and liraglutide) for the treatment of type 2 diabetic patients. Both exenatide and liraglutide stimulate insulin secretion, inhibit glucagon secretion and increase or sustain the level of GLP-1 in circulation via increased protection from DPP-IV degradation (the improved pharmacokinetic profile of liraglutide is discussed further in Section 1.4.3). Notably, clinical trials have demonstrated a suppression in food intake and appetite with exenatide and liraglutide in patients with type 2 diabetes (Monami *et al.*, 2009; Norris *et al.*, 2009 and Jendle *et al.*, 2009; Nauck and Marre, 2009, respectively), when used as monotherapy or as part of combination treatment. Regarding additional new pharmacotherapies licensed for obesity, the FDA has not approved a new prescription anti-obesity drug since 1999

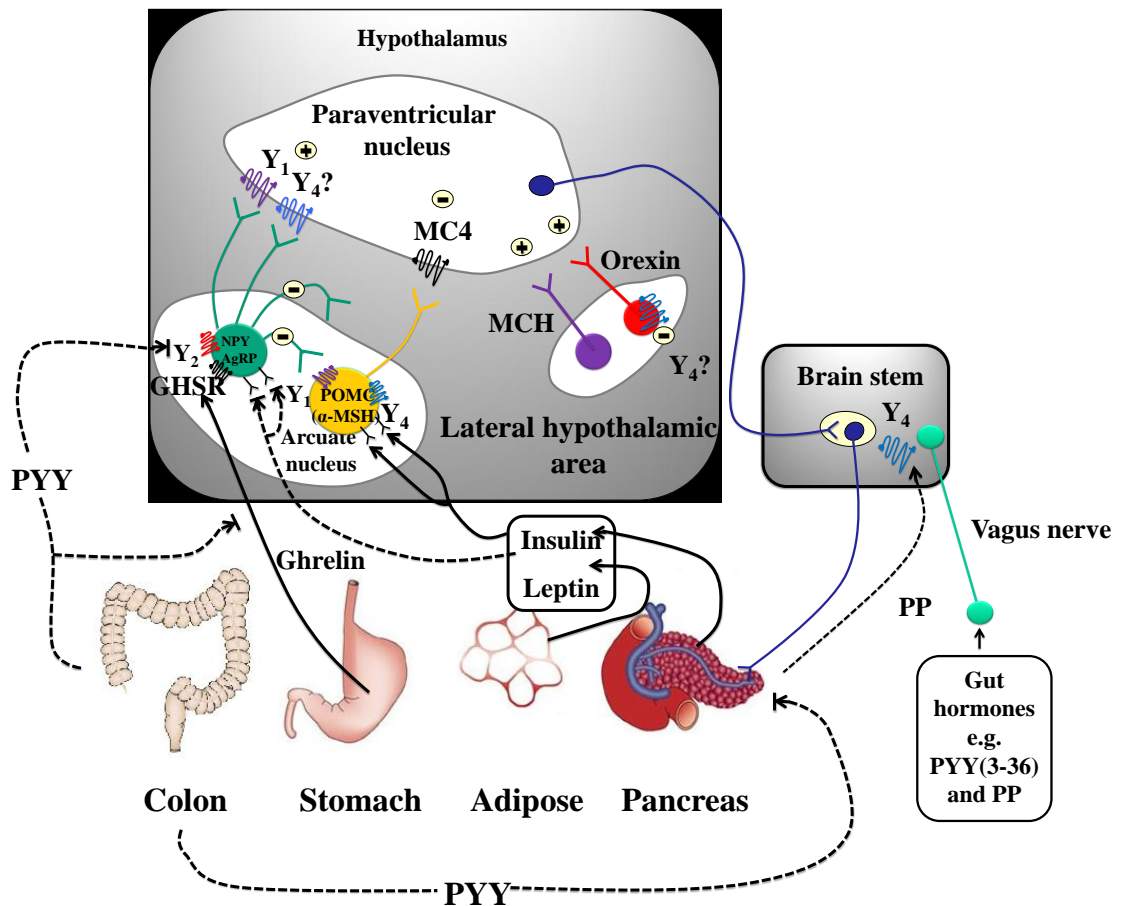
when it opened the US market to orlistat. Now more than ever, the development of new pharmacological strategies is crucial to tackle obesity alongside the adoption of a more active lifestyle.

### **1.3.2 Gut-brain axis and the regulation of food intake**

An extensive number of peripheral signals from the GI tract, liver, endocrine pancreas and adipocytes have the capacity to modulate energy intake and expenditure controlled in the CNS (Morton *et al.*, 2006; Cummings & Overduin, 2007). For these peripheral signals to influence central pathways in the regulation of food intake, 2 key transmission routes are involved; either they can cross the blood-brain barrier to elicit an effect directly on CNS neurones, or they can act upon vagal afferents. The brainstem and hypothalamus are the key CNS regions accountable for the regulation of energy homeostasis, receiving peripheral neural and hormonal signals regarding nutritional state and adiposity (Murphy and Bloom, 2006). It is the arcuate (ARC) nucleus of the hypothalamus which plays a central role on food intake and contains two populations of neurones with opposing effects (Chaudhri *et al.*, 2008). The medially located orexigenic (hunger-stimulating) neurones express NPY and Agouti-related protein (AgRP) (Broberger *et al.*, 1998; Bewick *et al.*, 2005). Opposing anorexigenic (satiety-stimulating) neurones in the lateral ARC express  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) derived from pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) (Elias *et al.*, 1998). Since the ARC borders the median eminence, a region which has partial blood-brain barrier protection (Peruzzo *et al.*, 2000), circulating peripheral hormones therefore have access and can manipulate ARC neurones directly. Evidence suggests GI hormones in response to food intake convey signals to the ARC to indicate short-term nutrient availability. On the contrary, peripheral factors such as insulin and leptin originating from the pancreas and adipose tissues, respectively, are thought to signal information corresponding to long-term energy stores (Porte *et al.*, 2002), both of which act on excitatory receptors on the POMC neurones to inhibit food intake and also inhibit the release of orexigenic NPY from the NPY/AgRP neurones (Niswender and Schwartz, 2003). The interactions between the brain and periphery are summarised in Figure 1.6. The stimulation of food intake occurs with an initial release of ghrelin, from endocrine cells of the oxyntic mucosa of the stomach (Tschop *et al.*, 2000; Wren *et al.*, 2001). Via activation of growth hormone secretagogue (GHS) receptors located on NPY and AgRP-containing neurones in the ARC (Nakazato *et al.*, 2001), ghrelin is able to cause release of NPY



which subsequently stimulates the orexigenic  $Y_1$  receptors in the paraventricular nucleus (PVN) (Broberger *et al.*, 1999). There is also some evidence which suggests that high levels of NPY may stimulate  $Y_4$  receptors located in the PVN (Parker and Herzog, 1999). Orexigenic signals are then relayed to the nucleus tractus solitarius (NTS) in the medulla, pituitary, limbic system, cortex and adrenal glands, resulting in food intake.



**Figure 1.6 The modulation of central pathways via peripheral factors to manipulate energy expenditure and food intake.** Ghrelin released from the oxyntic mucosa of the stomach activates NPY/AGRP neurones via the GHS receptor. NPY activates  $Y_1$  and possibly  $Y_4$  receptors in the PVN resulting in ingestion. Insulin and leptin stimulate their receptors on POMC neurones and inhibit NPY/AGRP neurones. In the lateral hypothalamic area (LHA), melanin-concentrating hormone (MCH) and orexin neurones stimulate the PVN. In addition, inhibitory  $Y_2$  receptors on NPY neurones of the arcuate nucleus may become activated by PYY released from distal colon, signalling satiety. Furthermore, PYY can also activate the ‘ileal brake’, contributing to satiety. PP release from the pancreas is also thought to inhibit appetite via the  $Y_4$  receptor in the ARC, LHA and brainstem. Dotted lines correspond to inhibitory pathways and solid lines correspond to stimulatory pathways. Adapted from Tough, 2005.

### 1.3.2.1 The role of PYY(3-36) in satiety

In 1985, Adrian *et al.* first demonstrated that circulating levels of PYY(3-36) rise within 15 minutes following food consumption, and remain elevated for up to 6 hours (Adrian *et al.*, 1985). The extent at which PYY(3-36) levels rise is proportional to the number of calories consumed (Degen *et al.*, 2005). Protein-rich meals in particular have been shown to heavily stimulate the release of PYY(3-36), compared to other macronutrients (Batterham *et al.*, 2006; Pedersen-Bjergaard *et al.*, 1996). In 1993, the gut hormone was first shown to reduce food intake, following peripheral administration of the peptide in rodents (Okada *et al.*, 1993). Batterham *et al.* then went on to successfully support these findings by demonstrating decreased appetite for a minimum of 12 hours with PYY(3–36) injections (Batterham *et al.* 2002). Although early studies could not replicate the acute inhibitory effects of PYY(3–36) on feeding in rodents, several studies have now reproduced and confirmed these findings in rodents (Abbott *et al.* 2005; Koda *et al.* 2005; Unniappan *et al.* 2006; Vrang *et al.* 2006; Unniappan & Kieffer, 2008), in non-human primates (Koegler *et al.* 2005) and also in humans (le Roux *et al.* 2006; Batterham *et al.* 2007; Sloth *et al.* 2007a; Sloth *et al.*, 2007b).

Strong evidence indicates that anorectic actions of PYY(3–36) are mediated via inhibitory pre-synaptic Y<sub>2</sub> receptors in the ARC. The fact that PYY(3–36) reduces NPY mRNA levels and release of NPY from *ex vivo* hypothalamic explants, further supports this hypothesis. Furthermore, concentrated patterns of *c-fos* immunoreactivity are highlighted in ARC, a region where Y<sub>2</sub> receptors are localised, following peripheral administration of PYY(3–36). Further evidence for the role of the Y<sub>2</sub> receptor in feeding has been provided by Batterham *et al.* who showed reduced food intake following peripheral PYY(3–36) is lost in Y<sub>2</sub> receptor knock out (KO) mice (Batterham *et al.* 2002). PYY(3-36) is thought to exert its effects through vagal brainstem mediated pathway; following bilateral sub-diaphragmatic total truncal vagotomy or transectioning of the brain stem hypothalamic pathway in rodents, the effects of PYY(3-36) on feeding (Abbott *et al.*, 2005; Koda *et al.*, 2005), and on ARC neuronal activation are lost (Abbott *et al.*, 2005), implicating the vagus nerve in the regulation of appetite.

Besides its centrally acting mechanisms, PYY(3–36) is also able to modulate appetite via effects on GI motility; PYY is able to exert inhibitory actions on gastric acid secretion, gastric emptying, and mouth-to-caecum transit time (Field *et al.*, 2010). With respect to upper small intestinal transit, PYY acts as an ‘ileal brake’, slowing intestinal

motility and resulting in satiety (Lin *et al.*, 1996; Maljaars *et al.*, 2008). Interestingly, obese individuals have lower levels of PYY(3–36) (Le Roux *et al.*, 2006), and its anorexigenic effect is preserved, unlike leptin, making the gut hormone a promising therapeutic target.

### 1.3.2.2 The role of PP in satiety

PP is released from the endocrine pancreas and like PYY, this process occurs in response to ingestion of a meal. Several studies have now successfully elucidated a role for PP in the anorectic response to feeding. Acute peripheral administration of the gut hormone significantly reduced appetite and food intake in both lean human subjects (Batterham *et al.*, 2003; Jesudason *et al.*, 2007) and obese patients with Prader Willi Syndrome (Berntson *et al.*, 1993; Glaser *et al.*, 1988; Gettys *et al.*, 1991). Using both freely fed and fasted mice, Balasubramaniam *et al.* (2006) also demonstrated a dose-dependent reduction in food intake following peripheral injection of PP. This reduction was  $Y_4$  receptor-mediated, since the effect was completely abolished in  $Y_4^{-/-}$  mice.

The anorectic effects of PP through  $Y_4$  agonism are mediated via both central and peripheral mechanisms. Central  $Y_4$  receptor expression was shown in the hypothalamic ARC of mice (Parker and Herzog, 1999; Parker and Herzog, 2000; Figure 1.6) and in 2003, Asakawa *et al.* demonstrated reduced mRNA expression of NPY and orexin in the hypothalamus of mice following repeated administration of PP over a 24 hour period (Asakawa *et al.*, 2003). In 2009, Lin *et al.* corroborated these findings by showing intense c-Fos activation in the ARC in response to *i.p.* injection of PP in wild type (WT) but not in  $Y_4^{-/-}$  mice, an effect that was particularly strong in neurones co-expressing  $\alpha$ -MSH. This group provided further evidence of central  $Y_4$  receptor activation via PP, since a number of other hypothalamic areas such as the PVN and ventromedial nucleus indicated c-Fos immunoreactivity following PP administration. Furthermore, *in situ* hybridisation experiments demonstrated an upregulation of POMC mRNA in the ARC (Lin *et al.*, 2009). To ascertain whether this effect was  $Y_4$ -specific, Lin *et al.* utilised hypothalamus-specific conditional  $Y_4^{-/-}$  mice and revealed a loss in both PP-induced c-Fos activation in the ARC and increased POMC mRNA expression, suggesting a central role for  $Y_4$  signalling.

Along with PP's central actions, the gut hormone has been reported to reduce appetite via a reduction in gastric emptying. In addition, PP has an inhibitory action on intestinal

and colonic motor activity and peristalsis (Fujimiya and Inui, 2000), an effect that is dependent on intact vagal signalling (Murphy and Bloom, 2006; Field *et al.*, 2010).

Encouraging data indicating PP's and PYY's anorectic actions led to 7TM Pharma (Copenhagen) to develop two synthetic analogues for the treatment of obesity: TM30339 and TM30338. Peripheral administration of the selective Y<sub>4</sub>-receptor agonist, TM30339, exhibited significant weight loss in diet-induced obese mice. This led to a 28 day human study in which safety, tolerability and the effective dose to induce weight loss were ascertained in healthy obese individuals (7TM Pharma meeting report, 2008). The novel Y<sub>4</sub> agonist was reported to be well tolerated and exhibited few side effects. TM30338, named Obinipitide, binds to both the hY<sub>2</sub> and the hY<sub>4</sub> receptor. Replacement of Pro at position 34 with glutamine (Gln) on the hPP amino acid sequence led to a dual agonist with nM potency at both Y receptors (Schwartz, 2006). In Phase I/II clinical trials, Obinipitide demonstrated a promising safety profile in patients with a body mass index (BMI) averaging 34 and was able to reduce ghrelin levels and inhibit food intake by ~10% when administered as a single daily subcutaneous dose (7TM Pharma meeting report, 2008). The most promising aspect of this novel peptide is that it lacks significant Y<sub>1</sub> receptor affinity, this reducing the possibility of any adverse cardiovascular events (Pedrazzini, 2004). Besides the potential synergistic efficacy, the Y<sub>2</sub>/Y<sub>4</sub> dual agonist does not induce the emesis previously observed with Y<sub>2</sub>-selective agonists, as suggested by the preclinical monkey studies described by 7TM Pharma (7TM Pharma meeting report, 2008).

### 1.3.3 Y<sub>2</sub>/Y<sub>4</sub> synergy

Since both the Y<sub>2</sub> and Y<sub>4</sub> receptors play a key role in regulation of appetite and adiposity, it is postulated that the dual peptide, Obinipitide, may evoke a synergistic action in terms of its satiety-inducing activity, compared with single Y<sub>2</sub> or Y<sub>4</sub> agonists. With the use of germline knockout mice, several groups have demonstrated marked synergy between the two Y receptors in terms of fat mass accumulation (Sainsbury *et al.*, 2003; Sainsbury *et al.*, 2006; Lee *et al.*, 2008). Dual germline deletion of the Y<sub>2</sub> and Y<sub>4</sub> receptor (Y<sub>2</sub><sup>-/-</sup>Y<sub>4</sub><sup>-/-</sup>) in mice revealed a greater reduction in fat mass compared to mice possessing single Y<sub>2</sub> and/or Y<sub>4</sub> deletions (Sainsbury *et al.*, 2003). Furthermore, the implications of single, double, or triple loss of Y<sub>1</sub>, Y<sub>2</sub>, or Y<sub>4</sub> receptors were assessed in terms of alterations in energy balance. A high-fat diet in wild type mice resulted in the onset of diet-induced obesity, but interestingly, this was not observed in Y<sub>2</sub><sup>-/-</sup>Y<sub>4</sub><sup>-/-</sup> mice.

Germline deletion of the  $Y_1$  receptor in combination with either the  $Y_2$  or  $Y_4$  receptor ( $Y_1^{-/-}$  or  $Y_1^{-/-} Y_2^{-/-}$  or  $Y_1^{-/-} Y_4^{-/-}$ ) led to exacerbated obesity symptoms. Additionally,  $Y_2^{-/-} Y_4^{-/-}$  mice displayed a resistance to the effects of obesity, thought to be related to a reduction in food intake and increased glucose tolerance. Interestingly, both wild-type and  $Y_1^{-/-}$  mice displaying diet-induced obesity demonstrated decreased hypothalamic POMC expression, but this was not the case in  $Y_2^{-/-} Y_4^{-/-}$  mice fed a high-fat diet, who appeared to be resistant to the effects of obesity. The authors concluded a synergistic action between the  $Y_2$  and  $Y_4$  receptor, since dual deletion of these receptors results in protection against obesity, partly via alterations in feeding and hypothalamic POMC expression (Sainsbury *et al.*, 2006). In addition,  $Y_2$  receptor knockout mice appear to have dismissed symptoms of the massive obesity normally observed in leptin-deficient *ob/ob* mice, although this is not the case with  $Y_4$  receptor knockout mice (Sainsbury *et al.*, 2002), signifying differential mechanisms of each receptor to modulate adiposity. To further support these results in *ob/ob* mice, Lee *et al* showed a significantly reduced body weight and fat mass in *ob/ob* mice possessing deletion of  $Y_2$  and  $Y_4$  receptors, and this effect was enhanced compared to *ob/ob* mice with single  $Y_2$  receptor deletion (Lee *et al.*, 2008). Since ablation of both  $Y_2$  and  $Y_4$  receptors synergistically reduces adiposity significantly more in obese *ob/ob* mice compared with their lean counterparts, it is predicted a novel dual  $Y_2/Y_4$  receptor analogue will induce considerably enhanced effects on appetite and food intake.

## **1.4 Use of PEGylation and lipidation to increase therapeutic potential of peptides**

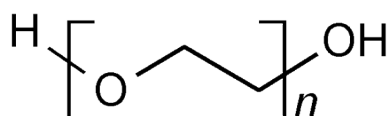
### **1.4.1 The shortcomings of PYY and PP as therapeutics**

The use of peptides and proteins as therapeutic agents has several drawbacks; their relatively small size makes delivery difficult and they are often cleared by the kidneys or mononuclear phagocyte system within minutes of administration (Pasut and Veronese, 2009). In addition, their susceptibility to degradation by proteolytic enzymes results in an exceptionally short half life. Adrian *et al* demonstrated that exogenously administered bPP is rapidly cleared from the circulation in humans with a half-life of 7.0 min, after infusion of 0.98 - 4.58 pmol/kg/min PP (Adrian *et al.*, 1978). Similarly, the circulating half life of PYY in plasma is 11 min in dogs (Pappas *et al.*, 1985). In rats, peak plasma levels of PYY(3-36) are reached at 15 min post injection, however in this study, the half-life was not reported (Batterham *et al.*, 2002). Despite the fact that

PYY(3–36) levels elevate rapidly after a test meal (4,500 kcal) and can remain elevated for 6 hours post meal (Adrian *et al.*, 1985), these levels unfortunately fall to baseline within 30 min when the peptide is infused (Batterham *et al.*, 2002). A number of research groups have attempted to improve the clinical properties of polypeptides via amino acid alteration, with the aim to protect against enzymatic degradation. Such methods have included polypeptide fusion to albumin, or incorporation of the peptides into drug-delivery vehicles such as liposomes (Mateo *et al.*, 2000; Lyczak & Morrison, 1994; Syed *et al.*, 1997). Although the use of liposomes has, in some cases, been successful, limitations such as rapid entry into the liver, spleen, kidneys and reticuloendothelial systems, as well as leakage of the drugs while in the circulation (Allen, 1997) have led to the need to additional drug delivery systems. PEGylation (the attachment of polyethylene glycol (PEG) to a peptide) and lipidation (acylation of a peptide with fatty acids) are alternative methods that overcome these deficiencies. In fact, liposomes are now PEGylated to improve the delivery of encapsulated drugs, such as the anticancer agent doxorubicin (Gabizon & Martin, 1997).

#### 1.4.2 PEGylation of peptides

The process of PEGylation is based upon the covalent linking of a PEG polymer to the core sequence of a peptide or macromolecule. Most commonly, the PEG exists in a linear or branched form with terminal hydroxyl groups, as depicted below in Figure 1.7.



**Figure 1.7 Structural formula of a PEG entity.**

For a stable reaction the PEG entity is frequently linked to a lysine, a known reactive group on the peptide. Other reactive linkers utilised in this process include glutamic acid, aspartic acid, free cysteine, threonine, the C-terminal carboxylic acid or the N-terminal amine of the peptide. The attachment of a PEG dramatically increases the overall molecular weight of the peptide, through binding to 2–3 water molecules per ethylene oxide unit (Figure 1.7). The flexibility of the PEG polymer in conjunction with the highly hydrated characteristic of the PEG-bound peptide allows the peptide to mimic a soluble protein which has a 5 to 10 times larger molecular weight compared

with the parent peptide. These properties of the PEG conjugate thus enable the PEG to precipitate proteins (Polson, 1977), reduce immunogenicity and antigenicity (Abuchowski *et al.*, 1977) and shield the peptide from proteolytic degradation. In addition, given that the renal glomeruli filter molecules based on their size, peptides conjugated to a PEG polymer exhibit reduced renal clearance. Thus, PEGylation offers a number of advantages which subsequently lead to an increased bioavailability and potentially the possibility of lower doses and less frequent administration. Furthermore, PEG induces a low risk of immunogenicity, even at high molecular weights (Caliceti and Veronese, 2003); to date there have been no reported situations in which the administration of a PEGylated molecule has led to the generation of anti-PEG antibodies in a clinical setting (Caliceti and Veronese, 2003).

An unintended outcome of PEGylation is a reduction, or sometimes even complete loss, in biological activity, thought to be a result of steric hindrance of the target site induced by the large PEG polymer. Techniques involving site-specific PEGylation have been described (Harris and Chess, 2003; King *et al.*, 1994), in which an unpaired cysteine linker is targeted by the PEG polymer and is engineered in such a way that it does not interfere with the binding motif. This approach has been successfully applied (Chapman *et al.*, 1999; Weir *et al.*, 2002), whereby it has been demonstrated that PEG chains with molecular weights of between 2 kDa and 40 kDa can be conjugated to antibody fragments without detrimental effect to their affinity. It should be noted however that conflicting reports exist with regards to the effects of PEGylation on affinity; in 2005, Kubetzko *et al.* site-specifically coupled a 20 kDa PEG moiety to the C terminus of an antibody construct, specifically to a cysteine residue separated by a glycine linker, to avoid PEG interference between the antibody and antigen interaction. A reduction in the functional affinity was reported when evaluating the binding properties of the PEGylated constructs to their unPEGylated equivalent. Examination of the binding kinetics revealed that the association rate between the PEGylated construct and the antigen were in fact slower, which was attributed to a combination of both intra-molecular/inter-molecular blocking mechanisms. Using Mass-transport limited BIAcore measurements, it was established that from the total number of PEGylated antibodies in solution at any given time point, only less than 7% were capable of interacting and binding to their target. The remaining PEG-antibodies in solution were considered to be intra-molecularly blocked in terms of binding. The authors concluded that although PEGylation led to a reduction in the association rate constant, all PEGylated antibody

constructs could ultimately successfully bind to their target, albeit with a slower equilibrium (Kubetzko *et al.* 2005). Although a reduction in *in vitro* binding affinity is often observed following PEGylation of a peptide, the marked increase in stability and prolonged half-life of the peptide *in vivo* is considered to more than compensate, since extended exposure and receptor occupancy are usually a consequence.

#### **1.4.2.1 PEGylated peptides currently in development**

Adagen, a PEGylated bovine adenosine deaminase, used for the treatment of Severe Combined Immunodeficiency Disease (SCID), was the first successful PEGylated pharmaceutical to be authorised by the Food and Drug Administration (FDA) in March 1990 (Chess and Harris, 2003). Subsequently, a number of PEGylated peptides moved swiftly into the clinic including Oncaspar, a PEGylated L-asparaginase responsible for the degradation of asparagines (Chess and Harris, 2003). In this case, PEGylation enhances the enzyme's circulating half life by 337 hours in addition to reducing the possibility of an immunogenic adverse reaction, thus making it an ideal treatment for leukaemia.

With obesity and type 2 diabetes becoming increasingly prevalent in the western countries, many attempts have been made to develop stable, long acting pharmacological drugs with promising selectivity. In 2006, Lee *et al.* applied the PEGylation technique to GLP-1, a therapy for type 2 diabetes, as a method to overcome the rapid clearance and inactivation of the peptide via DPP-IV (Kieffer *et al.*, 1995) and the neutral endopeptidase (NEP) 24.11 (Plamboeck *et al.*, 2005). Mono-PEGylation of a GLP-1 analogue (Lys-PEG/GLP-1) exhibited 40-, 28- and 10- fold enhanced stability in plasma, kidney and liver homogenates, respectively, compared with the native hormone. Furthermore, Lys-PEG/GLP-1 not only stimulated insulin release from rat pancreatic islets *in vitro* with a comparable potency to that of GLP-1, this also occurred in a glucose-dependent and concentration-dependent manner. Responses to glucose also appeared to stabilise following *i.p.* injection of Lys-PEG/GLP-1 (9 nmol/kg) in non-fasted *db/db* mice, and the PEGylated analogue displayed improved potency with regard to modulating glucose levels, compared with GLP-1 (Lee *et al.*, 2006). Pan *et al* engineered hybrid peptides which displayed both GLP-1 agonism and glucagon antagonism, as a result of targeted mutations. Site-specific PEGylation at the C terminus with either a 22 kDa or 43 kDa PEG-maleimide resulted in retention of *in vitro*



biological activity and a significantly prolonged mechanism of action *in vivo* (Pan *et al.*, 2006).

The success of PEG modified GLP-1 agonists led to the pursuit of long acting analogues of PYY and PP for potential treatments of obesity. In 2007, a novel Y<sub>2</sub> agonist was synthesised and conjugated with either a linear PEG (5 or 20 kDa) or branched PEG (40 kDa) at the N terminus via a Cys residue (DeCarr *et al.*, 2007). Administration of PEG5-modified PYY(24-36)-Leu<sup>31</sup> (5.6mol/kg) in mice induced a reduction in food intake, although this was not greater than that observed with PYY(3-36). Increasing the PEG size to 40 kDa (PEG40-PYY(24-36)-Leu<sup>31</sup>, 5.6mol/kg) resulted in inactivity *in vivo* within the first four hours of administration, however a small but statistically significant reduction in food intake of 12% and 8% was seen at 24 and 48 hours, respectively. Interestingly, an intermediary PEG size of 20 kDa (PEG20-PYY(24-36)-Leu<sup>31</sup>, 5.6mol/kg) led to comparable reduction in food intake to PYY(3-36), however unlike the native hormone, this effect was sustained from 4 to 24 hours and remained significant at 48 hours. Thus, this study demonstrated superior *in vivo* efficacy following a 20 kDa PEG modification of PYY(3-36) whilst a minimal effect feeding was observed after conjugation with a 40 kDa PEG, highlighting the significance of the PEG molecular weight in determining exposure at the drug target site. In addition, DeCarr *et al.* (2007) reported that the N-terminal modification of PYY(24-36)-Leu<sup>31</sup> was tolerated with a reduced EC<sub>50</sub> of approximately 6-fold. On the contrary, modification of the C-terminus of PYY(24-36)-Leu<sup>31</sup> was detrimental to *in vitro* receptor binding.

Consequently, Ortiz *et al.* (2007) synthesised a novel selective Y<sub>2</sub> receptor agonist, consisting of a peptide core PYY(13-36) and an N-terminally located non-peptidic moiety (2-mercaptanonicotinic acid) linked to a 20 kDa PEG polymer. The *in vitro* pharmacological profile was assessed and compared with a previously published 20 kDa PEG-PYY analogue with a shorter peptide core (PYY(25-36); Lumb *et al.*, 2007). Use of the longer peptide core corresponding to residues 13 to 36 of hPYY increased the *in vitro* Y<sub>2</sub> potency 4-fold compared with the original shorter PYY analogue, with K<sub>i</sub> and EC<sub>50</sub> values of 9.1 ± 2.2 and 6.5 ± 1.6 nM, respectively. Importantly, the longer peptide core did not exhibit high selectivity for the hY<sub>1</sub> and hY<sub>5</sub> receptors (K<sub>i</sub> = 760 ± 70 and 630 ± 30 nM, respectively). Additionally the peptide was able to bind rodent Y<sub>2</sub> receptors with similar affinity to the h receptor, with K<sub>i</sub> values of 21 ± 2 and 31 ± 6 nM

for the mouse (whole brain) and rat (hippocampus)  $Y_2$  receptors, respectively. Therefore, increasing the length of the peptide core improved *in vitro* receptor affinity while maintaining a high degree of specificity for the  $Y_2$  receptor. In lean C57BL/6 mice and Wistar rats, the PEG-modified PYY(13-36) analogue elicited a dose-dependent reduction in food intake which continued for 72 and 48 hours, respectively. Administration of the  $Y_2$  antagonist, BIIE0246, abolished the inhibition of food intake in lean C57BL/6 mice, providing evidence of  $Y_2$  selectivity with the PEGylated peptide. Daily dosing of the PYY analogue for 14 days in diet-induced obese (DIO) mice resulted in a dose-dependent reduction in body weight, which also persisted following dosing for 40 days (Ortiz *et al.*, 2007).

The data published by Lumb *et al.* (2007) and Ortiz *et al.* (2007) exemplifies a strategy to attain a series of selective  $Y_2$  receptor peptide agonists which have been modified by an N-terminally located non-peptidic moiety and a 20 kDa PEG polymer. The structural features of these peptides have included improved *in vitro*  $Y_2$  receptor potency (more so with the longer peptide core), enhanced  $Y_2$  receptor selectivity over the  $Y_1$  and  $Y_5$  as well as improved *in vivo* efficacy as a result of the PEG polymer conjugation. These groups have thus generated a selective  $Y_2$  peptide agonist which outperforms the clinical candidate PYY(3-36) in reducing food intake in both lean mice and lean rats following a single dose. Furthermore, the results provide preclinical evidence in two animal species that this peptide has potential for the management of obesity via selective activation of the  $Y_2$  receptor.

### 1.4.3 Lipidation of peptides

The principle behind lipidation involves the attachment of an acyl group to a peptide backbone; this provides it with the ability to non-covalently bind to plasma proteins, such as albumin and thus protect the peptide from enzyme degradation. Just as a PEG side chain sterically shields the peptide, the lipid moiety is thought to behave in the same way. The ability of a lipid moiety to facilitate plasma protein binding has been studied extensively by Kurtzhals *et al.* (1995). They investigated the binding of immobilised human serum albumin to various derivatives of lipidated insulin. It was reported that binding to albumin occurred with association constants of  $10^4$ - $10^5$  M<sup>-1</sup>, which interestingly was dependent on non-polar and ionic interactions and less so on lipid chain length.

Inspired by this, Bellman-Sickert *et al.* (2011) developed the first lipidated (and PEGylated) dual Y<sub>2</sub>/Y<sub>4</sub> analogue for the treatment of obesity. A Lys linker at position 13 was introduced to Obinepitide (TM30338) to enable site-specific modification with either a 2 or 20 kDa PEG polymer or a palmitoyl (Pam, C16) moiety, thus generating the peptides [K<sup>13</sup>(PEG2)Q<sup>34</sup>]hPP, [K<sup>13</sup>(PEG20)Q<sup>34</sup>]hPP and [K<sup>13</sup>(E-Pam)Q<sup>34</sup>]hPP. Competitive binding experiments with the non-modified peptide, [K<sup>13</sup>,Q<sup>34</sup>]hPP, revealed nanomolar affinities for both hY<sub>2</sub> and hY<sub>4</sub> receptor (IC<sub>50</sub> = 1.1 and 2.1 nM, respectively). Modification with the palmitoyl moiety was not detrimental to Y<sub>2</sub> receptor binding capacity (IC<sub>50</sub> = 0.6 nM) but a modest loss at the Y<sub>4</sub> receptor was reported (IC<sub>50</sub> = 26.0 nM). While modification with PEG2 did reduce the affinity marginally compared to the unmodified ligand (IC<sub>50</sub> was reported as 2.3 and 4.5 nM for Y<sub>2</sub> and Y<sub>4</sub>, respectively), increasing the PEG size (PEG20) dramatically decreased Y<sub>2</sub> and Y<sub>4</sub> receptor affinity (IC<sub>50</sub> = 34.0 nM and 231.0 nM, respectively). Proteolytic stability studies in human blood plasma also revealed increased resistance with the long chain fatty acid and large PEG side chain; [K<sup>13</sup>(PAM)Q<sup>34</sup>]hPP and [K<sup>13</sup>(PEG22)Q<sup>34</sup>]hPP both demonstrated approximately 10% degradation, compared with the non-modified hPP and [K<sup>13</sup>, Q<sup>34</sup>]hPP, both of which rapidly metabolised with a half life of 50 hours. Resistance to [K<sup>13</sup>(PEG2)Q<sup>34</sup>]hPP was observed but to a much lesser extent (% degradation not reported). The latter observation with [K<sup>13</sup>(PEG2)Q<sup>34</sup>]hPP is unexpected, as several groups have demonstrated a much higher stability with PEGylated peptides. For example, Na *et al.*, (2004) revealed no degradation of PEGylated salmon calcitonin (sCT) after incubation with nasal mucosa, compared with the unmodified sCT, which rapidly degraded by half with the observation period of 70 min. The data reported by Bellman-Sickert *et al.*, (2011) suggests that increasing the size of the PEG side chain may be detrimental to the receptor binding affinity and thus it is essential to determine the PEG size threshold at which the binding affinity is retained for the receptor.

Several groups have studied the effect of varying the fatty acid length on peptide stability; Yamamoto *et al.* (2003) observed dramatic stabilisation in skin homogenates following tripeptide acylation with increasing fatty acids, butyric, hexanoic, and octanoic acid. This data supports that of Bellman-Sickert *et al.* (2011) where little degradation was seen with the lipidated peptide. Importantly, Bellman-Sickert *et al.* also demonstrated prolonged anorexigenic effects with the lipidated hPP analogue. Administration of the unmodified [K<sup>13</sup>,Q<sup>34</sup>]hPP peptide (3 mg/kg) in mice significantly

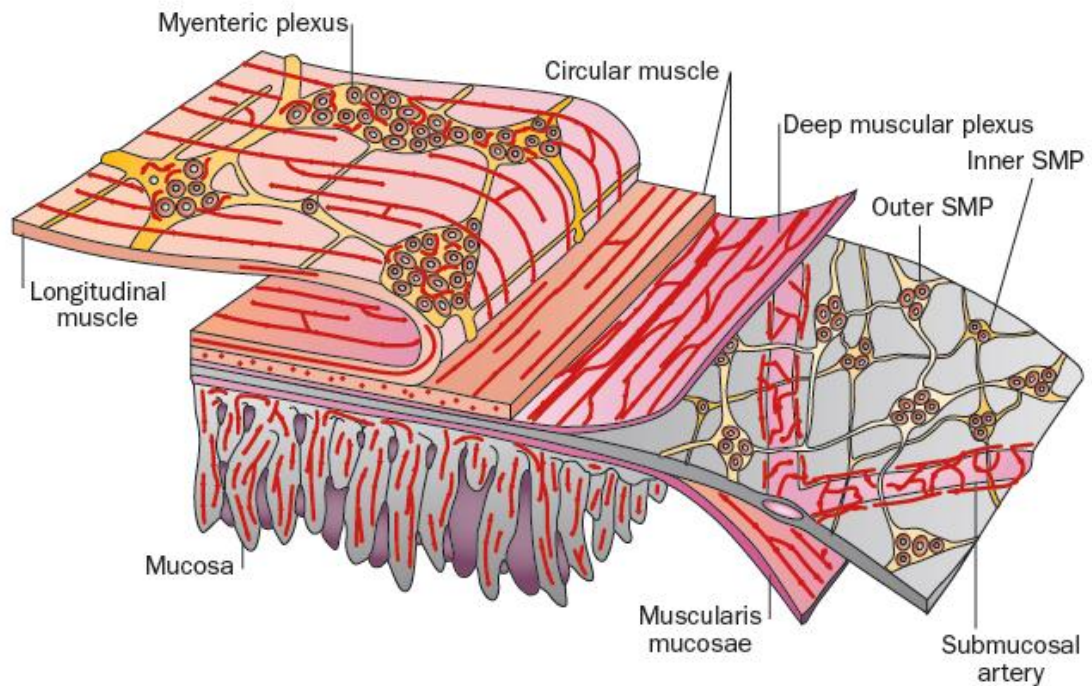
reduced food intake within 0.5 hours compared to vehicle treated mice. Although a reduction in food intake was not observed within 4.5 hours of administration of the palmitoylated variant, accumulated food intake remained lower compared to control mice throughout the total observation period of 12 hours. A further example of an improved pharmacokinetic profile obtained by successful fatty acid (C16) acylation is the successful GLP-1 derivative, liraglutide. Slow release from the injection site and increased protection from DPP-IV degradation are the two key benefits that the GLP-1 analogue has acquired through lipidation (Knudsen *et al.*, 2000). The increase in plasma stability (and thus half life) is substantial; subcutaneous injection of the peptide in pigs has shown an increase in plasma half life from 1.2 hours to 14 hours following chemical modification (Knudsen *et al.*, 2000). As a result, the peptide can now be used as a once daily subcutaneous injection in the treatment of type 2 diabetes (Ribel *et al.*, 2002; Arulmozhi and Portha, 2006).

As described above, significant advances have been achieved by the synthesis of peptides with additional chemical modifications to overcome limitations such as proteolytic susceptibility and limited bioavailability. PEG/lipid modifications have proved useful as tools in increasing the efficacy of novel peptide analogues and therefore may have promising therapeutic potential.

## **1.5 The role of the PP family in gastrointestinal functions**

### **1.5.1 Intestinal anatomy and coordination of the ENS**

Gastrointestinal function depends on the coordinated activity of enteric neurones within the GI tract (Bayliss and Starling, 1899). The ENS is composed of enteric ganglia, the neural connections between these ganglia, and nerve fibers that supply effector tissues, including the muscle of the gut wall, the epithelial lining, intrinsic blood vessels and gastroenteropancreatic endocrine cells (Figure 1.8). Two plexuses comprise the ganglia which are involved in the intrinsic innervations of the gut ENS (see section 1.1.5.3); the myenteric plexus (or Auerbach's plexus) and submucosal plexus (or Meissner's plexus). The former is situated between the outer longitudinal and the inner circular muscle layers and the latter is found between the muscularis mucosae and the circular muscle (Furness, 2000; Costa *et al.*, 2000; Grundy and Schemann, 2007).



**Figure 1.8 The organisation of the ENS of mammalian small intestine.** The ganglionated myenteric and submucosal plexus are situated between the longitudinal and circular layers of the external musculature and between the muscularis mucosae and the circular muscle, respectively. The two plexuses are interconnected via nerve fibre bundles which subsequently innervate the longitudinal and circular muscle layers, as well as the muscularis mucosae, intrinsic arteries and the mucosal lining (taken from Furness, 2012).

The ENS has multiple roles: determining the patterns of movement of the gastrointestinal tract; controlling gastric acid secretion; regulating movement of fluid across the lining epithelium; changing local blood flow; modifying nutrient handling; and interacting with the immune and endocrine systems of the gut (Furness, 2006). The ENS also contributes, along with glial cells, to maintaining the integrity of the epithelial barrier between the gut lumen and cells within the gut wall (Toumi *et al.*, 2003; Savidge *et al.*, 2007). At least 11 different types of neurones in the myenteric plexus of guinea-pig ileum have been identified based on their neurochemistries and projections (Costa *et al.*, 1996). If results from retrograde tracing of neuronal projections in the submucosal plexus are included, then 18 different neuronal subclasses can be identified in the guinea-pig ileum (Brookes, 2001). The neurones of the ENS can be split into four main categories: motor neurones supplying either the circular or longitudinal muscle layers, interneurones, intrinsic primary afferents (IPANs) and secretomotor neurones.

In the guinea-pig small intestine, the enteric inhibitory motor neurones supplying the circular muscle represent about 12% and the excitatory motor neurones innervating this

muscle layer are about 10% of the total number of myenteric neurones (Costa *et al.*, 1996; Grider *et al.*, 1998). Identification of transmitters used by enteric motor neurones advanced in parallel with the discovery that each class of enteric neurone may utilise multiple transmitters. This began with the identification of neurokinins (NK) as co-transmitters with acetylcholine (ACh) of the excitatory enteric motor neurones (Taylor and Bywater, 1983; Holzer *et al.*, 1993). Inhibitory neurones also express multiple transmitters with the relative importance of each varying with the region of the digestive tract and species; ATP, nitric oxide (NO), VIP, NPY and pituitary adenylyl cyclase activating peptide (PACAP) have all been identified as mediators of inhibitory neuromuscular transmission (Lecci *et al.*, 2002; Furness, 2006). VIP and PACAP are co-localised with NOS within inhibitory motor neurones (Brookes and Costa, 2002). This basic pattern holds true for most species including the guinea-pig, mouse, rat and human (Porter *et al.*, 1997; Sang and Young, 1998; Lomax and Furness, 2000).

In addition to the myenteric motor neurones, 16% of the myenteric nerves are interneurones that contain ACh co-released with various transmitters, depending on the function of the interneurone. For example, ascending interneurones involved in local reflexes co-release NK whereas descending local reflex interneurones co-release VIP/NOS/NPY/bombesin. Other myenteric interneurones are involved in secretomotor reflexes (containing ACh and 5-HT) or migrating myoelectric complexes (containing ACh and somatotropin release-inhibiting factor, SRIF) controlling mucosal ion transport and maintaining intestinal motility, respectively (Furness, 2000).

Sensation of the gut is transmitted via myenteric and submucosal IPANs or via extrinsic afferents from the spinal column. IPANs of the intestine are specialised neurones that encode information about the state of the intestine by transducing mechanical and chemical stimuli that reflect tension in the gut wall and the chemical nature of its contents. They connect with interneurones and motor neurones to form the circuits of intrinsic muscle motor, secretomotor and vasomotor reflexes. The IPANs are characterised by Dogiel type II morphology, with a slow after hyperpolarisation potential, which co-release ACh and NKs, although calcitonin gene-related peptide (CGRP) has also been identified in mouse colon (Furness *et al.*, 2004). Following activation, IPANs release substance P (Kirchgessner *et al.*, 1992) which stimulates NK receptors on submucosal neurones and leads to activation of secretomotor neurones. Stimulation of this intramural pathway leads ultimately to release of VIP and ACh onto

epithelial receptors resulting in  $\text{Cl}^-$  secretion e.g. as demonstrated in guinea pig colon (Weber *et al.*, 2001). Activation of IPANs and extrinsic primary afferents also results in release of SP and activation of excitatory smooth muscle motor neurones e.g. as observed in guinea pig ileum (Bartho *et al.*, 1994; Bartho *et al.*, 1999).

Enteric reflexes, through activation of secretomotor neurones, move water and electrolytes from the interstitium of the lamina propria to the lumen, thereby controlling epithelial ion secretion. Enteric secretomotor reflexes also modulate blood volume and blood pressure detectors that change the activity of two sympathetic pathways; vasoconstrictor pathways and secretomotor inhibitory pathways (Furness, 2006). The enteric receptor for glucose, composed of  $\text{T1R}_2$  and  $\text{T1R}_3$  subunits, is located on enteroendocrine L cells (Shirazi-Beechey *et al.*, 2011). Stimulation of these cells releases the endocrine hormones: GLP-1 and GLP-2, oxyntomodulin and PYY. Receptors for GLP-2 are on noncholinergic secretomotor neurones, which are activated by this hormone (Sigalet *et al.*, 2007; Shirazi-Beechey *et al.*, 2011). Thus, activation of the enteric receptor by glucose stimulates secretomotor neurones to return water and electrolytes to the lumen. Other endocrine products of L-cells, such as PYY, are involved in a range of functions, including modulation of fluid secretion, inhibition of gastric emptying, promotion of satiety, stimulation of epithelial cell growth and regulation of insulin secretion (Estall and Drucker, 2006; Sigalet *et al.*, 2007; Cox, 2007). The modulation of ion secretion by PYY and other members of the PP family is discussed below (see section 1.5.3.2).

### **1.5.2 Smooth muscle contraction**

Reports suggest that the PP family can modulate smooth muscle contraction. The majority of studies examining the effects of PYY on colonic motor function have been performed *in vitro* (Ferrier *et al.*, 2000; Hyland *et al.*, 2003; Pheng *et al.*, 1999; Sawa *et al.*, 1995); electrically stimulated contractions in colonic muscle strips of guinea pig were inhibited following superfusion of PYY (Sawa *et al.*, 1995) although PYY has also been shown to induce an elevation in basal contraction *in vitro* in longitudinal muscle from mice (Hyland *et al.*, 2003), rats (Ferrier *et al.*, 2000; Ferrier *et al.*, 2002; Pheng *et al.*, 1999), and human (Ferrier *et al.*, 2002) colonic tissue. In 2011, Tough *et al.* demonstrated transient longitudinal muscle contractions after the addition of PYY (30 nM) or NPY (100 nM) to WT mouse ascending colon. These contractions were unaffected in  $\text{NPY}^{-/-}$ ,  $\text{PYY}^{-/-}$  and  $\text{NPYPYY}^{-/-}$  tissue. Basal tension or spontaneous

activity were unaltered in the presence of either BIBO3304 nor BIIE0246, indicating a lack of endogenous contractile tone in WT colon, however BIIE0246 did inhibit the subsequent response to PYY and a similar inhibition was seen with both  $Y_1$  and  $Y_2$  receptor antagonism. This data corroborated previous studies showing PYY(3–36)-mediated contraction in  $Y_2^{+/+}$  colonic muscle, but not in  $Y_2^{-/-}$  tissue (Hyland *et al.*, 2003). Studies in mice (Hyland *et al.*, 2003) and rats (Feletou *et al.*, 1998; Ferrier *et al.*, 2000; Pheng *et al.*, 1999) have revealed colonic longitudinal smooth muscle contractions in response to PP. In 2010, Moriya *et al.* demonstrated a dose-dependent increase in faecal weight following peripheral administration of PP in mice, and that faecal output in response to PP was almost completely inhibited in  $Y_4^{-/-}$  mice. The data published by Moriya *et al.* was supported by a previous report (Wager-Page *et al.*, 1993) in which an increase in colonic intra-luminal pressure was observed with intravenous (*i.v.*) bolus injections of PP in anaesthetised rats, indicative of colonic contractions. Furthermore, PP-induced colonic transit may be used to explain an early study in which genetically obese *ob/ob* mice exhibited diarrhoea after chronic PP treatment (Mordes *et al.*, 1982). To summarise, PYY and PP coordinate changes in the patterns of smooth muscle contraction in wild-type colon via stimulation of  $Y_2$  and  $Y_4$  receptors. Given that Y receptors are pivotal in intestinal absorption (see section 1.5.3), it is thought that these contractions play a modulatory role in the final integrated intestinal response.

### 1.5.3 Epithelial ion transport

#### 1.5.3.1 Physiology of colonic epithelial ion transport

The key function of the proximal colon is absorption of salt and water. Bulk transport of NaCl in the colonic epithelium is due to electroneutral absorption by luminal  $Na^+/H^+$  and  $Cl^-/HCO_3^-$  exchange. The remaining absorption is electrogenic and is due to absorption via luminal epithelial  $Na^+$  channels and transcellular/paracellular absorption of  $Cl^-$  (Kunzelmann and Mall, 2002).

Electrogenic  $Na^+$  absorption is present throughout the human colon. The hallmark of this process is the presence of epithelial  $Na^+$  channels (ENaCs). ENaCs, comprised of  $2\alpha/2\beta/1\gamma$  subunits (Canessa *et al.*, 1995) are located apically in colonic epithelial cells, through which 3  $Na^+$  ions are exchanged for the basolateral uptake of two  $K^+$  ions, mediated via the ouabain sensitive electrogenic  $Na^+$  pump ( $Na^+/K^+$ ATP-ase), resulting



in the net transfer of one positively charged  $\text{Na}^+$  ion across the basolateral membrane.  $\text{Na}^+$  ions diffuse into the cell along a favourable electrochemical gradient. This gradient reflects the low potential difference across the basolateral membrane exceeding that across the apical membrane, resulting in a substantial lumen negative transmucosal potential difference in healthy human colon *in vivo* and *in vitro*, which largely reflects electrogenic  $\text{Na}^+$  transport (Sandle et al., 1986). An interesting feature of the electrogenic  $\text{Na}^+$  absorptive process in high transepithelial resistance epithelia is the extreme sensitivity to micromolar concentrations of the diuretic, amiloride and related diuretic compounds (Canessa *et al.*, 1994; Barbry and Hofman, 1997). In general, apical addition of amiloride produces  $\text{Na}^+$  channel blockade, inhibition of electrogenic  $\text{Na}^+$  absorption, and a reduction or abolition of the transepithelial potential difference. In the human colon, the nature and distribution of apical  $\text{Na}^+$  conductances, and their responses to amiloride, is complex; under *in vitro* conditions, addition of 0.1–1.0 mM amiloride to distal colon decreases the potential difference by 61–94% and the short circuit current ( $I_{\text{sc}}$ : an indicator of net transepithelial ionic transport when the potential difference is electrically “clamped” to zero) by 76–93%, whereas in proximal colon the electrical changes are minimal (Sandle et al., 1986).

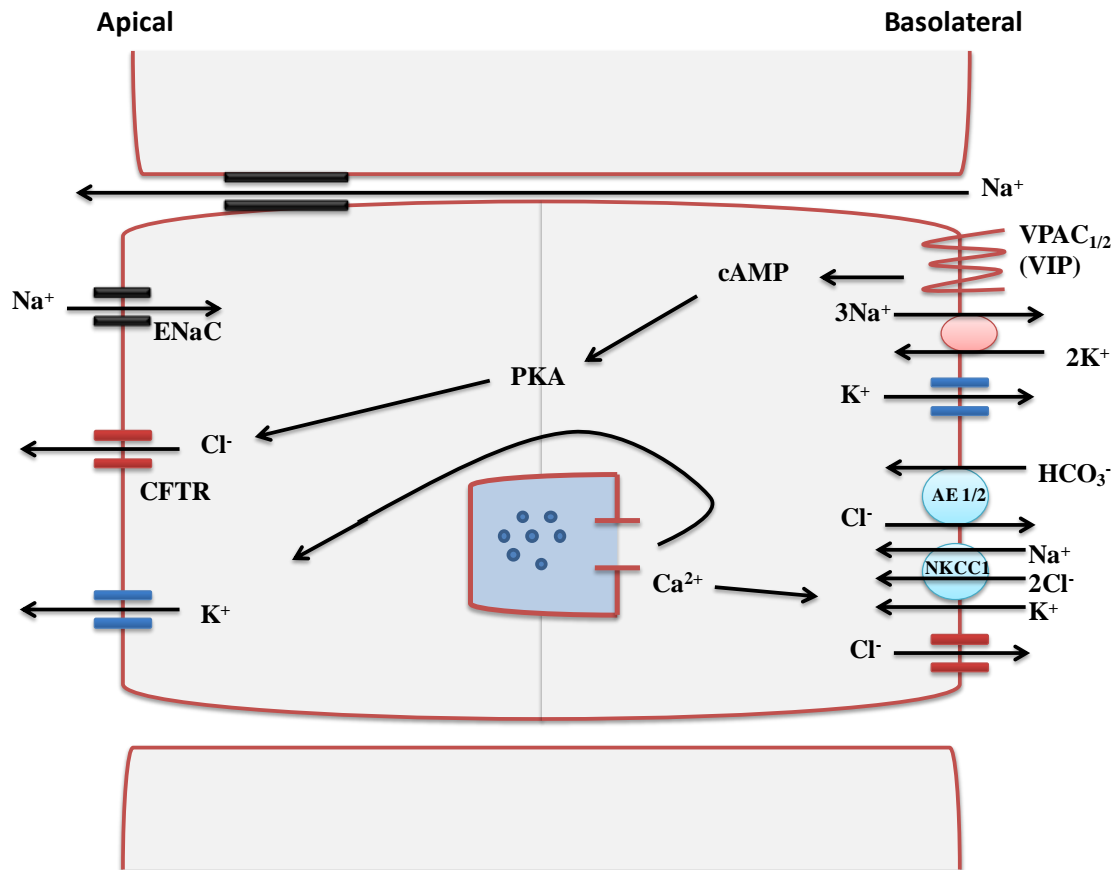
In parallel, the basolateral loop diuretic (bumetanide) -sensitive  $\text{Na}^+/\text{K}^+ / 2\text{Cl}^-$  co-transporter (NKCC1) allows the re-entry of  $\text{Na}^+$  into the cell, thus resulting in an increase in intracellular  $\text{Cl}^-$  concentrations. The increase in intracellular  $\text{Cl}^-$  subsequently inhibits the activity of the ENaCs, leading to a reduction in  $\text{NaCl}$  absorption (Konig *et al.*, 2001). Furthermore, basolateral  $\text{K}^+$  channels work to rectify increased intracellular  $\text{K}^+$  levels; the PKA-activated  $\text{K}_v\text{LQT1}$  channels and  $\text{Ca}^{2+}$  activated intermediate conductance  $\text{K}^+$  (IK) channels, through which  $\text{K}^+$  ions are recycled and hyperpolarise the cell, maintain  $\text{Cl}^-$  secretion.

Studies in isolated sheets of human sigmoid colonic and rectal mucosa have shown that 1 mM amiloride applied apically, decreases the  $I_{\text{sc}}$  to a far greater extent than net  $\text{Na}^+$  absorption, which suggests that a substantial fraction of net  $\text{Na}^+$  absorption is mediated by a process other than amiloride-sensitive electrogenic  $\text{Na}^+$  transport (Rask-Madsen and Hjelt, 1977). It is also clear from other studies that net  $\text{Na}^+$  absorption exceeds the  $I_{\text{sc}}$ , and there is considerable net  $\text{Cl}^-$  absorption in the proximal, transverse and distal colonic segments (Sellin and De Soignie, 1987). These findings suggest that an electroneutral  $\text{Cl}^-$  dependent  $\text{Na}^+$  absorptive process is present in all segments of the

colon. Furthermore, net  $\text{Cl}^-$  absorption and net  $\text{Na}^+$  absorption are equal and in part regulated by intracellular pH, as both are inhibited by acetazolamide, a carbonic anhydrase inhibitor that reduces endogenous  $\text{HCO}_3^-$  production (Foster *et al.*, 1983). In crypt and surface epithelia,  $\text{Na}^+$  is taken up from the apical side of the epithelium by type 2 and 3  $\text{Na}^+/\text{H}^+$  exchangers (NHE2 and NHE3). So far, three types of NHE in colonic epithelia have been detected. The profuse type 1 NHE is localised to the basolateral membrane and NHE2 and NHE3 are both expressed on the apical side of colonic epithelial cells, and it is thought that under control conditions, NHE3 contributes largely to the  $\text{Na}^+$  absorption (Ikuma *et al.*, 1999). Electroneutral  $\text{NaCl}$  absorption reflects apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange; functional studies in epithelia have demonstrated the existence of two apical and one basolateral  $\text{Cl}^-/\text{HCO}_3^-$  exchangers, the downregulated adenoma (DRA) protein and the anion exchanger (AE). AE1 exists on the apical domain, whereas the AE2 protein exists basolaterally.

Secretion and absorption are controlled by endocrine, paracrine, autocrine, immunologic, and neuronal stimuli. The major neuronal impact is due to the myenteric and submucosal plexi which innervate both epithelial and vascular smooth muscle cells, thereby controlling intestinal blood flow, secretion, and absorption in the colonic mucosa (Keast, 1987; Suprenant, 1994). Secretion of electrolytes is evoked by a variety of secretagogues, such as acetylcholine (Mall *et al.*, 1998), vasoactive intestinal polypeptide (VIP; Fuchs *et al.*, 1996), prostaglandins (Berschneider *et al.*, 1988) and inflammatory mediators from enteroendocrine cells e.g. 5-HT (Borman and Burleigh, 1996), as demonstrated in isolated human colonic mucosal preparations. One of the most prominent secretagogues, VIP, acts via increases in intracellular cAMP (Barrett and Keely, 2000); activation of the epithelial  $G_s$  coupled VPAC receptors leads to an elevation in the intracellular cAMP levels and hence an increase in protein kinase A (PKA) activity. As a result, PKA-sensitive CFTR channels, located apically, become phosphorylated and subsequently open (McDonald *et al.*, 1995), resulting in  $\text{Cl}^-$  secretion. Ion transport studies in CFTR<sup>-/-</sup> mice or cystic fibrosis (CF) patients have helped elucidate the importance the CFTR channel in electrogenic  $\text{Cl}^-$  secretion and have demonstrated the necessity of functional CFTR channels in agonist-induced secretion. In CFTR<sup>-/-</sup> mice, no secretory responses was observed to forskolin-mediated AC activation (Cuthbert *et al.*, 1994a), guanylate cyclase activation (Cuthbert *et al.*, 1994b) or increased intracellular  $\text{Ca}^{2+}$  levels by carbachol (Cuthbert *et al.*, 1994b) or lysylbradykinin (Cuthbert and Huxley, 1998) with isolated colon. Furthermore, human

colon mucosa from CF patients demonstrated no response to cAMP-elevating agonists or muscarinic  $\text{Ca}^{2+}$ -mediated responses (Berschneider *et al.*, 1988). The process of epithelial electrogenic anion secretion is outlined in Figure 1.9.



**Figure 1.9 Cellular model for electrogenic  $\text{Cl}^-$  secretion in response to VIP-stimulated mammalian colon.**  $\text{Na}^+$  is taken up from the apical side of the epithelium by epithelial ENaCs. The basolateral  $\text{Na}^+$ - $\text{K}^+$ -ATPase generates the driving force for the apical  $\text{Na}^+$  uptake by lowering intracellular  $\text{Na}^+$  concentration.  $\text{Na}^+$  re-enters the cell via the basolateral bumetanide-sensitive  $\text{Na}^+$ / $\text{K}^+$ /2  $\text{Cl}^-$  co-transporter (NKCC1), thereby increasing intracellular  $\text{Cl}^-$  concentrations. Recycling of  $\text{K}^+$  taken in via the NKCC1 transporter occurs via  $\text{Ca}^{2+}$ -activated intermediate conductance  $\text{K}^+$  (IK) channels.  $\text{VPAC}_{1/2}$  receptor activation results in a rise in intracellular cAMP, thus leading to an increase in PKA activity. The apical PKA-sensitive CFTR channel is subsequently activated, leading to  $\text{Cl}^-$  ions exiting the cell via this route (Harmar *et al.*, 2004; Banks *et al.*, 2005). The increased intracellular cAMP also enhances basolateral  $\text{K}^+$  exit by activating  $\text{K}_v\text{LQT1}$  channels resulting in a continuous hyperpolarised membrane potential and the driving force for secretion of  $\text{Cl}^-$  out of the cell. Adapted from Kunzelmann and Mall (2002).

The secretory action of hormones and neurotransmitters is balanced by the inhibitory effects of neuropeptides, endogenous opiates, noradrenaline and growth hormones. Amongst these include the PP family of peptides, whose inhibitory actions on ion secretion have been described below.

### 1.5.3.2 The ability of the PP family to inhibit of ion transport in colonic epithelia

Under voltage clamp conditions, electrogenic ion transport in polarised epithelial sheets or isolated mucosa can be measured, from which a resulting  $I_{sc}$  can be recorded (for details see Chapter 2). This can be achieved with the Ussing chamber, which was originally developed in 1951 to investigate  $Na^+$  absorption in frog skin (Ussing and Zerahn, 1951). It has been well documented that each member of the PP family can reduce  $Cl^-$  secretion in a number of rodent and human models, as demonstrated in colonic epithelial preparations, either in culture or with muscle-stripped mucosa. Friel *et al.* (1986) were the first to report the antisecretory effects of NPY and PYY in guinea pig and rabbit ileum; this group demonstrated reduced  $Cl^-$  transport from serosa to mucosa in the presence of NPY. This was later corroborated by Cox *et al.* (1988) who showed NPY-mediated reductions in  $I_{sc}$  and  $Cl^-$  secretion in muscle-stripped rat jejunum and descending colon, however only basolateral additions of PYY and NPY were able to inhibit the  $I_{sc}$ . The authors reported that this effect was dependent upon endogenous eicosanoid formation since it was blocked by the cyclooxygenase inhibitor, piroxicam, reducing electrogenic chloride secretion presumably as a consequence to elevated  $I_{sc}$  levels. The authors also demonstrated that NPY responsiveness could be restored, in piroxicam pre-treated tissues, or by VIP, forskolin, prostaglandin E2 (PGE2), isobutyl-1-methyl-xanthine (IBMX) and dibutyryl cAMP added prior to the neuropeptide. Several groups have now provided strong evidence for the inhibitory actions of the PP family in rabbit (Hubel and Renquist, 1986), guinea pig (McCulloch *et al.*, 1987), rat isolated intestinal mucosa (Cox and Cuthbert, 1988; Cox and Cuthbert, 1990; Strabel and Diener, 1995; Tough and Cox, 1996; Nakanishi *et al.*, 1996; Whang *et al.*, 1997) and mouse mucosa, where  $Y_1$ ,  $Y_2$  and  $Y_4$  receptors are found (Cox *et al.*, 2001a; Cox and Tough, 2002). Extensive studies have also been performed using human colon mucosa, demonstrating the anti-secretory response to  $Y_1$ ,  $Y_2$  and  $Y_4$  receptor activation (Cox and Tough, 2002). The authors determined that only anti-secretory responses to the  $Y_2$  responses were sensitive to the effects of tetrodotoxin (TTX), in contrast to both the  $Y_1$  and  $Y_4$  receptors, which were unaltered, demonstrating that the  $Y_2$  receptor type is predominantly pre-junctional i.e. on neurones whereas the  $Y_1$  and  $Y_4$  receptors are post-junctional i.e. on epithelia.

In 1985 the human colonic adenocarcinoma (HCA) cell line, HCA-7, was first established. Due to the heterogenous nature of this cell line, nine subpopulations in total

were isolated based on their morphology using cloning cylinders (Marsh *et al.*, 1993). Of the nine subpopulations, three were found to constitutively express Y receptors: Colony 1 (Col-1; Y<sub>4</sub>), Colony 6 (Col-6; Y<sub>1</sub> and Y<sub>4</sub>) and Colony 24 (Col-24; Y<sub>4</sub>). Once grown to confluence on semi-permeable supports, they form a polarised sheet (Kirkland, 1985), and ion transport studies can subsequently be performed. RT-PCR performed by Cox *et al.* demonstrated that Col-24 and to a lesser extent Col-6 cells express the Y<sub>4</sub> receptor in contrast with HT-29 negative controls that Y<sub>4</sub> receptor expression (Cox *et al.*, 2001b). Functional ion transport studies with Col-24 and Col-1 cells by the same group demonstrated receptor localisation at the basolateral domain, since basolateral additions of PP (following stimulation with VIP; Holliday *et al.*, 1997; Cox *et al.*, 2001b) were effective, in contrast to significantly smaller reduction in I<sub>sc</sub> when the peptide addition was made to the apical compartment. Furthermore, PYY was ineffective in either Col-24 or Col-1 indicating Y<sub>4</sub> expression only, whereas Col-6 cells exhibited sensitivity to hPP, PYY, NPY and Pro<sup>34</sup>NPY, demonstrating both Y<sub>1</sub> and Y<sub>4</sub> receptor expression (Cox and Tough, 1995). Responses to hPP in either Col-6 or Col-24 cells were unaltered in the presence of the Y<sub>1</sub> antagonist BIBP3226; however PYY and Pro<sup>34</sup>NPY responses in Col-6 cells were attenuated by BIBP3226 (Tough and Cox, 1996) indicating predominant Y<sub>4</sub> receptor activation with hPP in Col-24 cells. Different species PP stimulated responses in Col-24 with Y<sub>4</sub>-like pharmacology. bPP, hPP and pPP were equipotent while rPP, avian (a)PP and (Pro<sup>34</sup>PYY) were significantly less potent. Homologous PP desensitisation was also observed in Col-24 cells within 20 min of adding the first addition of PP, indicating that the different species PP (bPP, hPP and pPP) were activating the same population of Y<sub>4</sub> receptors. Therefore, these functional ion transport studies have demonstrated Y<sub>4</sub> receptor expression in Col-24 and Col-1 cells, and both Y<sub>1</sub> and Y<sub>4</sub> receptor expression in Col-6 cells.

## 1.6 The GIPIO collaboration

This project was funded by the European Union's Seventh Framework Programme (Grant Agreement No. 223057) and coordinated by University of Leipzig. The GIPIO (Gastrointestinal Peptides in Obesity) collaboration was set up with EU (FP7) funding in 2008 in which a consortium of 4 academic institutions (Universities of Leipzig, Copenhagen and Dresden, and King's College London) and 3 medium sized pharmaceutical companies (7TM Pharma, Zealand Pharma and PolyPeptide) were involved. The focus of this collaboration was to determine the contributions of key GI peptides in the regulation of food intake, specifically PP and PYY, with the aim to

develop potential anti-obesity agents that mimicked these endogenous gut hormones. With the promising results of Obinipitide and TM30339 in preclinical and clinical trials (7TM Pharma, 2008), the collaboration aimed to modify and optimise these peptides (via PEGylation and lipidation) to improve their metabolic stability and pharmacokinetic profile. A series of peptide variants based on the parent peptides (Obinipitide and TM30339) were subsequently produced by synthetic approaches (Bellmann-Sickert *et al.*, 2011) and the investigation of these novel peptides at the molecular, cellular, tissue and whole animal levels was performed.

## 1.7 Aims

The aim of this PhD project within the GIPIO collaboration was to provide the *in vitro* pharmacological data to aid the progression of the most promising peptide candidates. The initial aims of this study were to characterise and compare Y receptor-mediated anti-secretory responses to novel dual Y<sub>2</sub>/Y<sub>4</sub> and single Y<sub>4</sub> receptor agonists in an attempt to establish the consequence of chemical modification on the pharmacological profiles *in vitro*, using an optimised Y<sub>4</sub> expressing cell line (Col-24) and human colon mucosa, expressing both Y<sub>2</sub> and Y<sub>4</sub> receptors (as well as Y<sub>1</sub>). We sought to establish whether PEGylation and/or lipidation, of different size and chain length, respectively, would prolong the longevity of action compared with the native hormones, and whether potency and efficacy would be altered by these modifications. During the course of the study, we also set out to ascertain the receptor types mediating the anti-secretory response in human mucosal tissue, using selective Y<sub>1</sub> and Y<sub>2</sub> antagonists to establish the selectivity of the novel analogues. Having established the pharmacology, we investigated whether PEGylation or lipidation of the peptides altered subsequent Y<sub>4</sub> receptor desensitisation, compared with their non-modified predecessors.

## **2 METHODS**

## 2.1 Materials

### 2.1.1 Peptides

All commercially available peptides, unless stated otherwise, were acquired from Bachem Laboratories Inc. (Merseyside, U.K.). Once diluted in distilled H<sub>2</sub>O, subsequent aliquots were frozen at -20°C and underwent one freeze-thaw cycle prior to use. In this study, the porcine peptide sequence of VIP and the human sequences of PP, PYY and somatostatin (14-28) were used. hPP and all hPP analogues were synthesised and provided by Prof. A. Beck-Sickinger's group (University of Leipzig, Germany) and have been listed in Table 2.1.

Parent peptide	Amino acid alteration	Chemical conjugation	Receptor preference
<b>hPP</b>			Y <sub>4</sub>
<b>Obinepitide (TM30338)</b>	Q <sup>34</sup>	[Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
	K <sup>30</sup> , Q <sup>34</sup>	[K <sup>30</sup> (E-Pam)Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
	K <sup>30</sup> , Q <sup>34</sup>	[K <sup>30</sup> (PEG2)Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
	K <sup>30</sup> , Q <sup>34</sup>	[K <sup>30</sup> (PEG5)Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
	K <sup>30</sup> , Q <sup>34</sup>	[K <sup>30</sup> (PEG22)Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
	K <sup>22</sup> , Q <sup>34</sup>	[K <sup>22</sup> ,Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
	K <sup>22</sup> , Q <sup>34</sup>	[K <sup>22</sup> (PEG5)Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
	K <sup>22</sup> , Q <sup>34</sup>	[K <sup>22</sup> (PEG22)Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
	K <sup>22</sup> , Q <sup>34</sup>	[K <sup>22</sup> (E-Pam)Q <sup>34</sup> ]hPP	Dual Y <sub>2</sub> /Y <sub>4</sub>
<b>TM30339</b>	hPP <sub>2-36</sub>	hPP <sub>2-36</sub>	Y <sub>4</sub>
	[K <sup>22</sup> ]hPP <sub>2-36</sub>	[K <sup>22</sup> ]hPP <sub>2-36</sub>	Y <sub>4</sub>
	[K <sup>22</sup> ]hPP <sub>2-36</sub>	[K <sup>22</sup> (E-Capr)]hPP <sub>2-36</sub>	Y <sub>4</sub>
	[K <sup>22</sup> ]hPP <sub>2-36</sub>	[K <sup>22</sup> (E-Laur)]hPP <sub>2-36</sub>	Y <sub>4</sub>
	[K <sup>22</sup> ]hPP <sub>2-36</sub>	[K <sup>22</sup> (E-Pam)]hPP <sub>2-36</sub>	Y <sub>4</sub>
	[K <sup>22</sup> ]hPP <sub>2-36</sub>	[K <sup>22</sup> (PEG22)]hPP <sub>2-36</sub>	Y <sub>4</sub>

**Table 2.1 Summary of the PEG/lipid-modified peptides based on the human PP sequence and their Y<sub>2</sub> and/or Y<sub>4</sub> receptor preferences.** The colours used above are those adopted in the results section for specific analogues. Caprylic acid (C8), lauric acid (C12) and palmitic acid (C16) have been abbreviated to E-Capr, E-Laur and E-Pam, respectively, and these abbreviations have been used throughout this thesis.



### 2.1.2 Non-peptides

The Y<sub>1</sub> and Y<sub>2</sub> receptor antagonist, BIBO3304 and BIIE0246, respectively, were provided by Boehringer Ingelheim Pharma KG (Biberach an der Riss, Germany). Each antagonist was diluted in dimethyl sulphoxide (DMSO) (10%), aliquoted, stored at -20°C and discarded 48 hours after one freeze-thaw cycle. UK 14,304 (5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine), the α<sub>2</sub>-adrenoreceptor agonist (diluted in 10% DMSO) was purchased from Research Biochemical International (Natick, MA, U.S.A.). The apical Na<sup>+</sup> channel blocker, amiloride, was purchased from Sigma (Poole, U.K.), and the NKCC1 blocker, bumetanide, was purchased from Alexis Biochemicals (Nottingham, U.K.). PEG polymers and fatty acids (caprylic acid, lauric acid and palmitic acid) were purchased from Sigma-Aldrich (Poole, U.K.). Other additional reagents were sourced from Sigma (Poole, U.K.).

## 2.2 Human tissue collection and preparation

Human specimens taken from the distal colon were attained from patients undergoing resection surgery for primary carcinoma. Colonic specimens were taken from one of two procedures: (i) anterior resection or (ii) right hemi-colectomy. 24 hours prior to each procedure, informed written consent was obtained, along with ethical approval for the use of human tissue by The Guy's and St Thomas' Hospital Ethical Research Committee. Only gender, age, and procedure type was recorded in this study, and all other data remained anonymous, including previous or current medical history. Following excision from the patient, colonic segments were dissected (with a minimum margin of 10 cm from the carcinoma) by a consultant histopathologist. The specimen was then placed in Krebs-Henseleit solution (KH, composition in mM: NaCl 118, KCl 4.7, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5, glucose 11.1; pH 7.4) until muscle dissection. Using a dissection blade and a wax dissection bed, the mucosa was removed from the overlying smooth muscle layers and then divided into similar-sized, adjacent preparations. Each mucosal piece was subsequently mounted between 2 halves of an individual Ussing chamber with an exposed area of 0.64cm<sup>2</sup> or 0.14cm<sup>2</sup>, depending on the amount of mucosa available per specimen. Both the basolateral and apical sides were kept bathed in KH (5 ml, 37°C) and continually aerated with 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>.

### 2.3 Cell culture

Col-24 cells are derived from a HCA-7 cell line (Marsh *et al.*, 1993) and once grown to confluence, the cells are able to form a polarised epithelial monolayer. Col-24 cells were maintained as described previously (Cox and Tough, 1995); cells were optimally grown at 37°C in 25 cm<sup>2</sup> Falcon flasks (Beckton Dickinson, U.S.A.), and kept in a humidified atmosphere of 95% O<sub>2</sub>/5% CO<sub>2</sub>. Dulbecco's modified Eagle's medium (DMEM; Invitrogen, U.K.) supplemented with 10% foetal calf serum (FCS) (Invitrogen, Paisley, U.K.), glucose (25 mM), and the antibiotics kanamycin (100 µg ml<sup>-1</sup>) and amphotericin B (1.2 µg ml<sup>-1</sup>) (purchased from Sigma, Poole, U.K.), was used to maintain the cells and replaced on a twice weekly basis. Upon becoming confluent (within 6 days), the cells were cleansed with 10 ml versene followed by trypsinisation (0.5 % w/v in versene; Worthington Biochemical Corporation, Lorne Labs, U.S.A.). To maintain growth, cells were split at a ratio of 1:5. Only Col-24 cells between passages 11-24 were used in this study; additional stocks at a low passage were frozen in liquid nitrogen (with supplemented DMEM containing 10 % DMSO), for use in later experiments.

### 2.4 Preparation of epithelial monolayers

For ion transport studies, Col-24 cells were grown onto Millipore HAWP filters (25 mm diameter, pore size 0.45 µm). The filters were prepared by coating with collagen (0.25 % w/v solution of Type VII from rat tail in 0.2 % acetic acid) and air-dried at room temperature for 2 days prior to use. The collagen ensured that the cells grew basolateral side down. Once dry, a sylgard ring (2 mm thick with central well area of 0.2 cm<sup>2</sup>) was attached to each filter using a silicone adhesive, and each filter was placed under a short wave ultraviolet lamp and sterilised with at least 24 hour irradiation. Once each filter was prepared, Col-24 epithelia in 25 cm<sup>2</sup> flasks were trypsinised (as described above), and re-suspended in 2.4 ml DMEM. 100 µl of cell suspension (~ 2 x 10<sup>6</sup> cells) was then seeded into the central well of each filter. 4 seeded filters were then placed into in 90 mm petri dishes (Falcon, Beckton Dickinson, Oxford, U.K.) and suspended onto 12 ml DMEM. The filters were maintained under the same conditions as the Col-24 flasks. Epithelial layers reached confluence within 6 days, after which they were mounted in Ussing chambers and ion transport studies were carried out at 6, 7 and 8 days.

## 2.5 Measurement of electrogenic ion transport

Human intestinal sections were orientated as a flat sheet to separate two halves of a perspex Ussing chamber and intestinal preparations or Col-24 epithelial monolayers were situated vertically such that the apical membrane (also referred to as the luminal-side membrane) faced one chamber half, whereas the basolateral membrane (also referred to as the blood-side membrane) faced the other half-chamber, thus separating the KH buffer that independently bathed each chamber half. The Ussing chambers used had an exposed apex area of  $0.2\text{ cm}^2$  for Col-24 monolayers or  $0.64\text{ cm}^2$  and  $0.14\text{ cm}^2$  for human tissue. Reservoirs above each chamber were water jacketed to enable warming of KH buffer to  $37^\circ\text{C}$  for the human preparations. Each reservoir was filled with 5 ml (human mucosa) or 15 ml (epithelial monolayers) of oxygenated (95%  $\text{O}_2$ /5%  $\text{CO}_2$ ) KH buffer.

Under the above conditions, the intestinal mucosa or epithelial monolayer exhibited a spontaneous transepithelial voltage potential ( $V_t$ ), measured by electrodes connected via salt bridges to each chamber half. The preparations were then voltage-clamped at 0 mV (as shown in Figure 2.1) using a DVC1000 voltage-clamp unit (World Precision Instruments, Stevenage, U.K.) as described in detail previously (Cox *et al.*, 1988). Voltage clamping the potential to zero eliminated the passive transepithelial driving force created by the electrical potential across the epithelium. Thus, the potential difference was negated on both sides and the current flowing through the short-circuit was therefore a result of the net active transport of ions across the epithelia (Ussing and Zehran, 1951). The resulting short-circuit current ( $I_{sc}$ , measured in  $\mu\text{A}$  per unit area) was monitored on an LCD screen on the DVC1000 and recorded continuously on a chart recorder (Kipp and Zonen, Lincoln, U.K.). A reduction in  $\text{Cl}^-$  secretion (from basolateral to apical domain) was measured as a reduction in  $I_{sc}$ .

Once mounted in chambers and voltage clamped, preparations were left to stabilise until a stable basal  $I_{sc}$  was achieved (normally 30-60 min for human tissue and 10-20 min for epithelia). A 1mV pulse was applied at the start of each experiment and the resultant  $I_{sc}$  deflection was used to calculate the transepithelial resistance (TER, measured in  $\Omega\cdot\text{cm}^2$ ) using Ohms law ( $\text{TER}=\text{voltage}/\text{current}$ ), providing an indication of the confluence of each preparation. At least one preparation was used as a control within each set of experiments to counteract the variability between adjacent sections of human tissue.

Due to  $G_i$  coupling of the receptors, activation of the Y receptors leads to a reduction in cAMP-dependent  $Cl^-$  secretion. At the start of each experiment all Col-24 monolayers were pre-treated with VIP (30 nM) since activation of the  $G_s$  coupled VPAC1 receptors causes an elevation in  $I_{sc}$ , which remains raised for at least 15 min. Once a maximal level of secretion was achieved, the addition of a Y agonist was made and a reduction in  $I_{sc}$  was observed (see Chapter 3, Figure 3.1 for a trace representing changes in  $I_{sc}$  to VIP and hPP). Since basal currents were high in human mucosa preparations, mucosal preparations were not pre-stimulated with VIP, thus demonstrating a more physiological situation. Where the effects of the  $Y_1$  and  $Y_2$  receptor antagonists, BIBO3304 (300 nM) and BIIE0246 (1  $\mu$ M), were investigated on subsequent agonist responses in mucosal preparations, the equilibration period was at least 20 min prior to addition of agonists. Either somatostatin (100 nM) or the  $\alpha_2$  adrenoceptor agonist, UK 14,304 (1  $\mu$ M) were used to verify the anti-secretory capability of the preparation following the Y agonist response in Col-24 monolayers or human mucosa, respectively. All additions of agonists, antagonists and bumetanide (200  $\mu$ M) were made to the basolateral reservoir, whereas amiloride (10  $\mu$ M) was added to the apical reservoir.

**Figure 2.1 The Ussing chamber system.** Mucosal sheets of human colon or Col-24 epithelial monolayers were placed between the two halves of the Ussing chamber, bathed with KH buffer on either side. Voltage electrodes, one on either side of the preparation ( $V_{ap}$  and  $V_{bl}$ ), measured the transepithelial voltage and the DVC1000 imposed an equal and opposite voltage, set at 0 mV, which voltage-clamped the tissue. The tissue was short-circuited by injection of a current, adjusted via a feedback amplifier to keep the transepithelial voltage ( $V_{te}$ ) at 0 mV, and the resulting  $I_{sc}$  (measured in  $\mu A$ ) was continuously recorded on a chart recorder. The system was configured so that a reduction in  $Cl^-$  secretion from the basolateral to the apical side was measured as a decrease in  $I_{sc}$ . This schematic has been adapted from Tough (2005).

### **2.5.1 Measurement of time-dependence of agonist responses**

In Col-24 monolayers each filter was pre-treated with VIP (30 nM) for 15-20 min and once a stable maximal  $I_{sc}$  was achieved, peptide analogues were added to the basolateral reservoir. The time-dependence of the Y receptor analogues were then determined where agonist responses were measured for 45 min in Col-24 monolayers and 60 min in human colon mucosa, at a concentration of 100nM. In the event of a waning  $I_{sc}$  after VIP pre-treatment, responses were measured by drawing a straight line on the trace

which followed the  $I_{sc}$  5 min prior to agonist additions. The line was extrapolated over the duration of the agonist response profile and the maximal difference between the extrapolated line and the  $I_{sc}$  was taken as the peak response. Time course profiles were constructed by pooling the individual observations at each time point after agonist addition ( $t=1, 2, 3, 4, 5, 10$ , and then every 5 min for the duration of the response). All values were then expressed as the mean  $\pm$  1 S.E.M., in  $\mu A.cm^{-2}$  over a period of 45 or 60 min.

### **2.5.2 Concentration-response relationships**

Non-cumulative concentration response curves were analysed by non-linear regression using the curve-fitting software, GraphPad Prism (V.501, San Diego, CA), to establish  $EC_{50}$  values (with 95% confidence limits). For short-acting peptides, peak responses were generally achieved within 5 min. For longer acting peptides where responses were slower to peak, values were taken at approximately 45 min after addition or the time at which the maximum reduction in  $I_{sc}$  was observed.

### **2.5.3 Measurement of $Y_4$ receptor desensitisation**

$Y_4$  receptor desensitisation was assessed by measuring the response to a near-maximal single concentration of hPP (10 nM) added either 25 min after an initial addition of hPP (10 nM) or another short-acting agonist, or in the case of long-acting  $Y_4$  agonists (100 nM), at 45 min. In human colon mucosa where reductions in  $I_{sc}$  were slower to peak, the response to hPP (100nM) was measured 60 min after an initial addition of a dual  $Y_2/Y_4$  or  $Y_4$  agonist (100nM).

### **2.5.4 Data analysis and statistics**

Pooled data from Ussing chamber studies are quoted as  $\mu A.cm^{-2}$ , mean  $\pm$  1 S.E.M.

#### **2.5.4.1 Concentration response curves**

$Y$  agonist concentration-response curves were constructed from single peptide additions to mucosa or epithelial monolayers. Statistical significances between each analogue in terms of maximal response size were determined using one-way analysis of variance (ANOVA) with either Dunnett's or Bonferroni's post-tests where appropriate. Single comparisons were made using Student's unpaired  $t$ -test. In all cases a  $P$  value of less than 0.05 was considered significant. For each curve the best fit was achieved by fixing

the minimum value response to 0  $\mu\text{A}$ , while all other parameters, such as curve maxima and Hill slopes were allowed to vary, unless stated otherwise.

#### **2.5.4.2 Y<sub>4</sub> Desensitisation**

Multiple comparisons of data groups were made using ANOVA with either Dunnett's or Bonferroni's post-tests where appropriate and single comparisons were made using Student's unpaired *t*-test. In all cases a *P* value of less than 0.05 was considered significant.

### **3 RESULTS**



### 3.1 General observations

#### 3.1.1 Col-24 epithelial monolayers

##### 3.1.1.1 Comparison of electrical parameters

Transepithelial resistance and basal  $I_{sc}$  values were comparable to observations seen previously (unpublished data, Cox *et al.*). Once epithelial monolayers reached confluence within 6 days, they were mounted in Ussing chambers and ion transport studies were carried out for three consecutive days. A one-way ANOVA with a Bonferroni's post-test demonstrated no significant differences in the electrical parameters between 6, 7 and 8 days (Table 3.1).

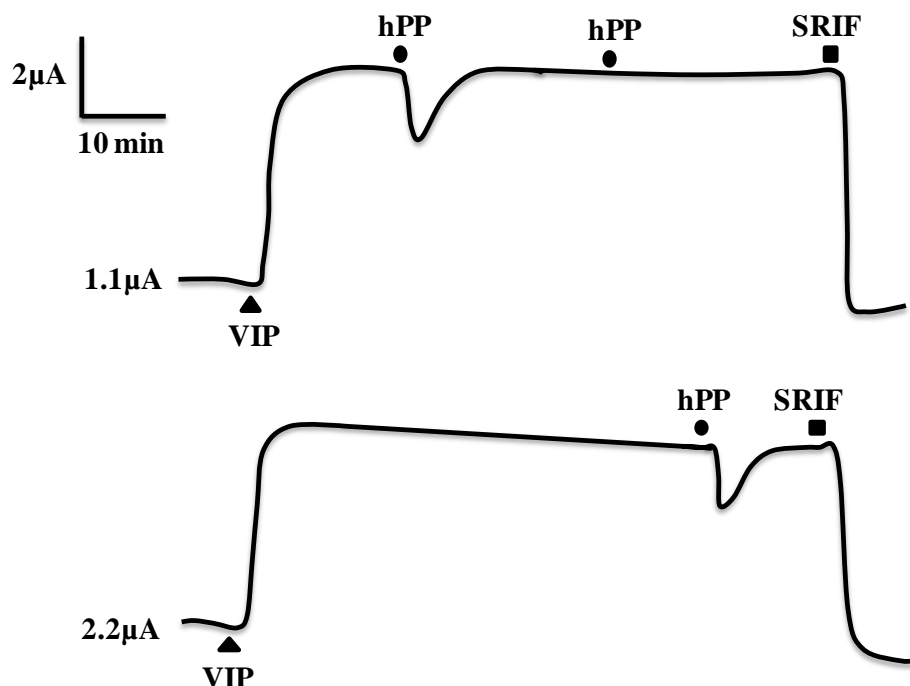
	Day 6	Day 7	Day 8
Resistance ( $\Omega \cdot \text{cm}^2$ )	$49.8 \pm 1.2$ (184)	$48.3 \pm 1.2$ (212)	$45.1 \pm 1.3$ (92)
Basal $I_{sc}$ ( $\mu\text{A} \cdot \text{cm}^{-2}$ )	$7.7 \pm 0.4$ (184)	$8.4 \pm 0.4$ (212)	$8.0 \pm 0.7$ (92)

**Table 3.1 Comparison of pooled transepithelial resistance and basal  $I_{sc}$  values in Col-24 monolayers.** Values in parenthesis denote the number of observations.

##### 3.1.1.2 Peptide responses in Col-24 monolayers

In each epithelial monolayer, VIP (30 nM) stimulated increases in  $I_{sc}$  (known to be due to cAMP-mediated apical chloride secretion) and peak responses occurred within 15 min (Figure 3.1, upper trace). Subsequent anti-secretory responses to hPP (10 nM) were observed as transient inhibitory responses, which reached their nadir within 5 min of addition and returned to the pre-inhibited  $I_{sc}$  within 15 min. A second addition of hPP (10 nM) demonstrated homologous desensitisation of the  $Y_4$  receptor. The level of secretion following VIP was maintained for at least 45 min (Figure 3.1, lower trace) and although VIP responses began to wain to pre-stimulation levels, the level of secretion was sufficient to see subsequent inhibitory responses to hPP at the later time point. No significant difference was observed between the size of the response to hPP at the maximum level of VIP stimulation ( $-2.7 \pm 0.2 \mu\text{A} \cdot \text{cm}^{-2}$ ,  $n=42$ ) compared with hPP at 45 min after VIP addition ( $-2.2 \pm 0.3 \mu\text{A} \cdot \text{cm}^{-2}$ ,  $n=6$ ), determined using a Student's *t*-test. A final addition of somatostatin (100 nM) was made to each epithelial monolayer since this well-documented anti-secretory agent has shown previously to inhibit colonic mucosal ion secretion in the mouse (Samson *et al.*, 2000; Cox *et al.*, 2001a; Hyland *et*

*al.*, 2003) and more importantly in Col-24 monolayers (Cox *et al.*, 2001b). The addition of somatostatin, following VIP and hPP, resulted in a sustained inhibitory response lasting 20-30 min (see first 10 min in Figure 3.1). This data was used to make an assessment of the anti-secretory capability of the epithelial preparations unrelated to  $Y_4$  receptor activation.

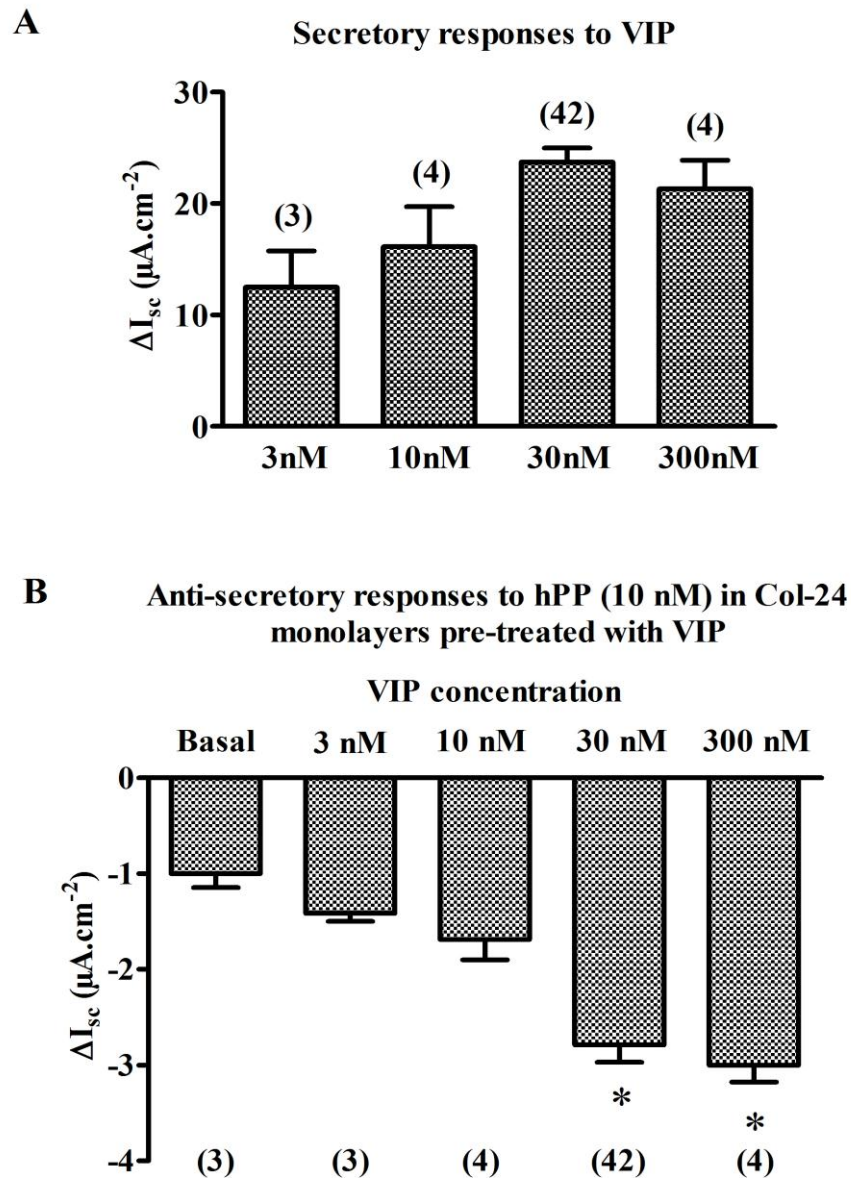


**Figure 3.1 Representative changes in  $I_{sc}$  following additions of VIP (30 nM), hPP (10 nM) and somatostatin (100 nM) in Col-24 monolayers.** The upper trace demonstrates the response to hPP at the maximum level of secretion after VIP pre-treatment. Subsequent  $Y_4$  desensitisation is induced by a second addition of hPP (10 nM). The lower trace illustrates that the level of secretion is maintained for at least 45 min following VIP, and that the response to hPP is comparable in size to that of hPP added at the earlier time point. Each monolayer received a final addition of somatostatin (100 nM), which completely inhibited the remaining VIP-elevated  $I_{sc}$ . Basal  $I_{sc}$  values are shown in  $\mu A$  to the left of each trace (note the exposed area was  $0.2 \text{ cm}^2$ ).

### 3.1.1.3 VIP responses in Col-24 epithelial monolayers

In 2001, Cox *et al.* demonstrated that  $Y$  agonist responses were dependent on the level of VIP stimulated  $I_{sc}$  in 129Sv mouse colon mucosa (Cox *et al.*, 2001a). In Col-24 epithelial monolayers, a single addition of hPP (10 nM, approximately the  $EC_{50}$  value) was added following increasing concentrations of VIP (3-300 nM). As VIP raised the  $I_{sc}$  in a concentration-dependent manner (Figure 3.2A), a concomitant increase in the hPP response (10 nM throughout) was observed with a maximum seen following 30 nM and

300 nM VIP (Figure 3.2B). All further Col-24 layers were therefore pre-treated with 30 nM VIP prior to the addition of either hPP or another  $Y_4$  agonist.



**Figure 3.2 Changes in  $I_{sc}$  after the addition of VIP and hPP in Col-24 monolayers.** VIP-induced increases in  $I_{sc}$  (3-300 nM) are shown in (A) and respective hPP-induced reductions (10 nM) in VIP pre-treated monolayers are shown in (B). Each bar represents the mean  $\pm$  S.E.M. of pooled responses. \*  $P < 0.05$ , compared with respective basal control hPP responses using one-way ANOVA with Dunnett's post-test.  $n$  values are shown in parenthesis.

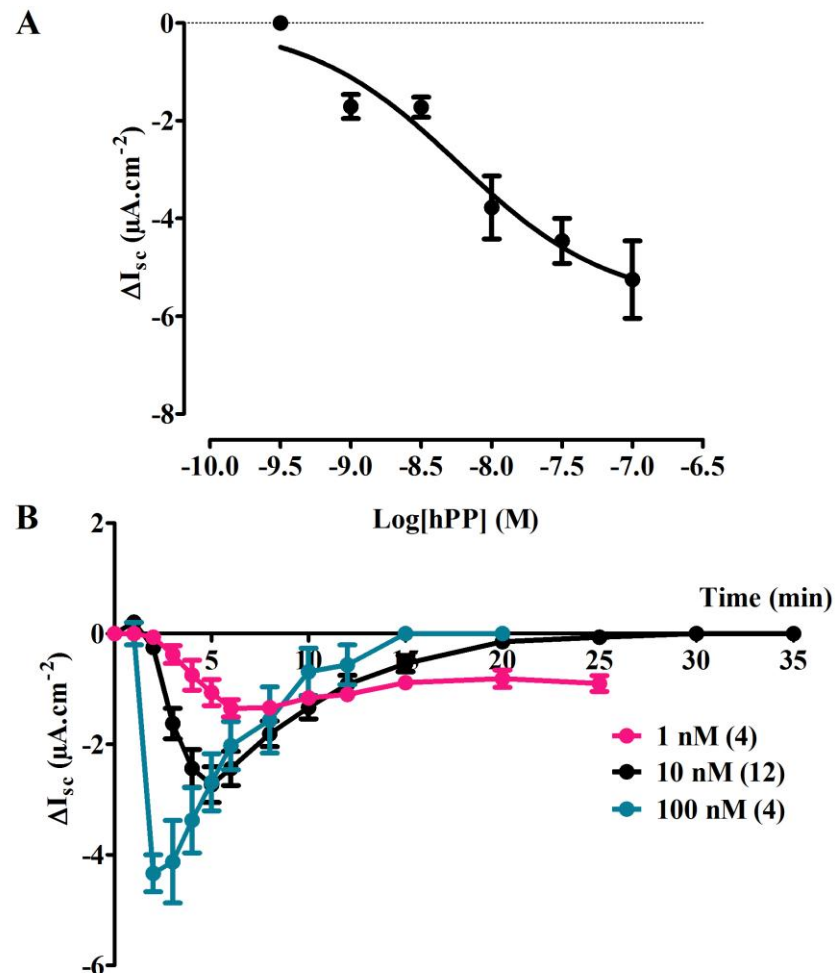
#### 3.1.1.4 Absence of any contribution of $Na^+$ absorption to the VIP $I_{sc}$ response

To establish whether epithelial  $Na^+$  channels contributed to the VIP-induced responses, the ENaC blocker, amiloride (20  $\mu M$ ) was added 15 min after VIP (30 nM). Amiloride had no effect on VIP stimulated  $I_{sc}$  values ( $16.4 \pm 2.4 \mu A.cm^{-2}$ ,  $n=4$ , prior to addition of amiloride and  $16.8 \pm 2.8 \mu A.cm^{-2}$ ,  $n=4$ ;  $P > 0.05$ , 10 min after addition of amiloride. In

contrast, bumetanide (200  $\mu\text{M}$ ), a NKCC1 blocker which prevents basolateral  $\text{Cl}^-$  entry (associated with sodium and potassium i.e. via NKCC1), significantly reduced VIP responses by 60% (from  $19.8 \pm 2.7 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=4$ , to  $7.9 \pm 2.4 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=4$ ;  $P < 0.05$ , within 5 min of addition). Thus VIP responses in Col-24 monolayers were not mediated by  $\text{Na}^+$  absorption through apical ENaC but were dependent upon basolateral  $\text{Cl}^-$  entry via the NKCC1 channel.

### 3.1.1.5 Responses to hPP in Col-24 epithelial monolayers

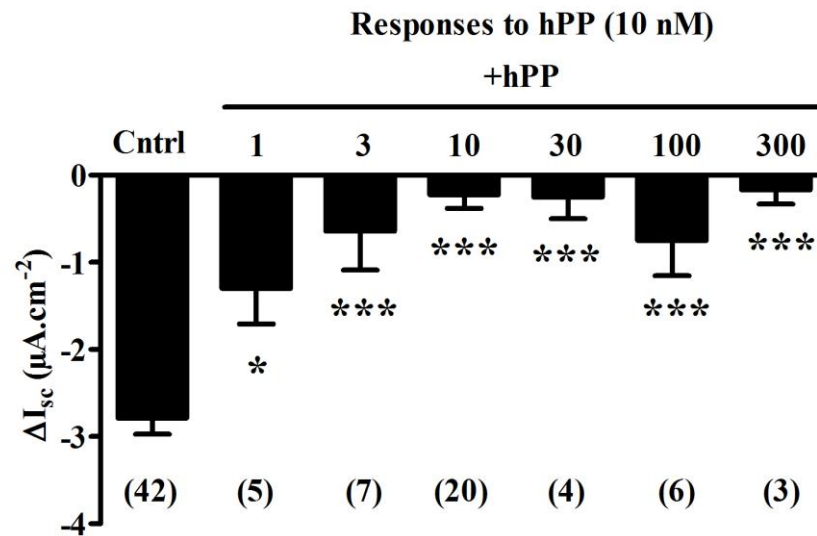
Responses to hPP (0.3-100 nM) were concentration-dependent and provided an  $\text{EC}_{50}$  value of 5.9 nM (1.0-34.0 nM) (Figure 3.3A). hPP-induced reductions in  $I_{\text{sc}}$  became more transient with increasing concentrations, and the time taken to reach peak response reduced from 6 min (1 nM) to 2 min (100 nM) (Figure 3.3B).



**Figure 3.3 Responses to hPP in Col-24 epithelial monolayers.** (A) Non-cumulative concentration-response curve for hPP after VIP (30 nM) pre-treatment. The curve generated the  $\text{EC}_{50}$  value quoted in the text. The data was pooled from 3-21 observations, with each point representing the mean  $\pm$  S.E.M. (B) A comparison of the time-course profiles for increasing concentrations of hPP (1 nM, 10 nM and 100 nM) over a 25 min period with  $n$  values indicated in parenthesis.

### 3.1.1.6 Desensitisation of hPP responses in Col-24 monolayers

In naive epithelial monolayers only pre-treated with VIP, hPP (10 nM) reduced the  $I_{sc}$  by  $-2.8 \pm 0.2 \mu A \cdot cm^{-2}$  ( $n=42$ ), however a second subsequent addition of hPP (10 nM, added 25 min after the first addition) demonstrated Y4 receptor desensitisation; the second addition reduced the  $I_{sc}$  by  $0.2 \pm 0.2 \mu A \cdot cm^{-2}$ ,  $n=20$ ,  $P < 0.001$  (a 92.9 % difference). This was a concentration-dependent effect, where increasing concentrations of the initial hPP addition caused further attenuation of the second hPP response (10 nM, Figure 3.4).



**Figure 3.4 Desensitisation of hPP-activated Y<sub>4</sub> responses in Col-24 monolayers.** Following pre-treatment of monolayers with increasing concentrations of hPP (1-300 nM), responses to a second addition of hPP (10 nM, shown above) demonstrated a concentration-dependent reductions in  $I_{sc}$  as the initial concentration of hPP increased. Each bar represents the mean - S.E.M with  $n$  values shown in parentheses. \*\*\*  $P < 0.001$ , \*  $P < 0.05$  compared with control hPP ('Cntrl', 10 nM) responses in naive monolayers, measured using one-way ANOVA and Dunnett's post-test.

### 3.1.2 Human colon mucosa

#### 3.1.2.1 Patient ages and mucosal electrical parameters

Transepithelial resistance and basal  $I_{sc}$  values of colonic mucosa (obtained from either anterior resection or right hemi-colectomy procedures) from male and female patients of a similar age are compared in Table 3.2. Since no statistical differences were observed between the electrical parameters, experimental data was pooled from both genders.

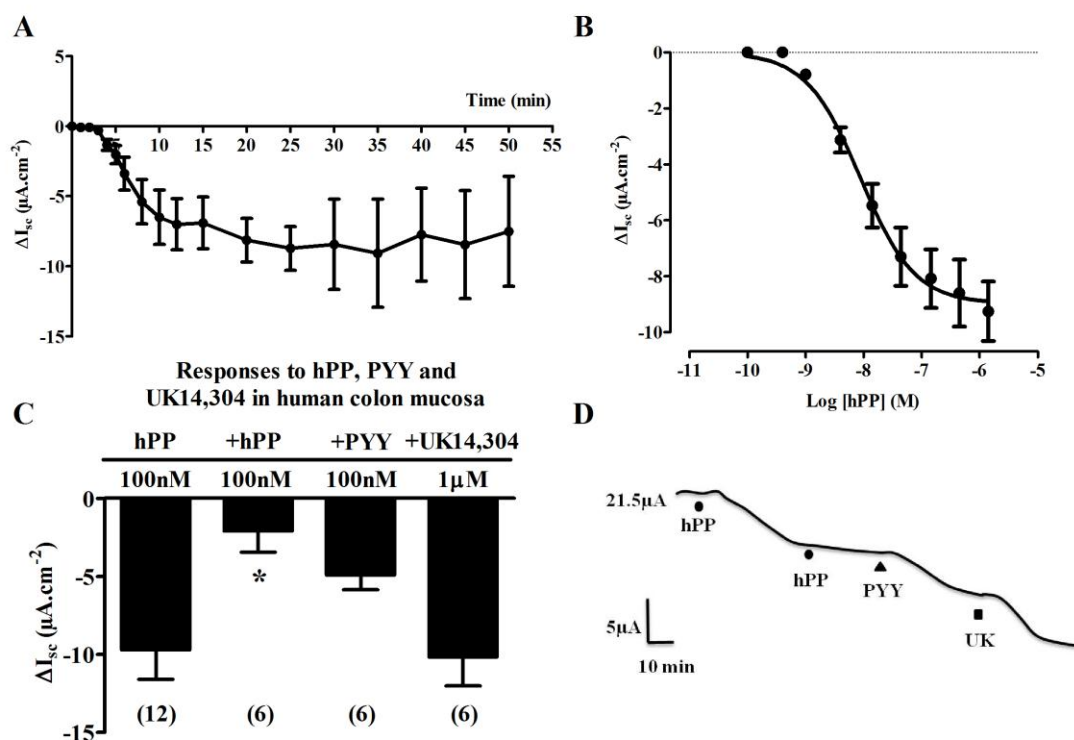
	Male	Female
Age (years)	59.0 $\pm$ 3.3 (10)	63.5 $\pm$ 5.3 (12)
Resistance ( $\Omega \cdot \text{cm}^2$ )	57.8 $\pm$ 5.1 (54)	52.4 $\pm$ 3.5 (72)
Basal $I_{sc}$ ( $\mu\text{A} \cdot \text{cm}^{-2}$ )	76.4 $\pm$ 6.6 (54)	90.0 $\pm$ 5.1 (72)

**Table 3.2 Comparison of ages, resistances and basal  $I_{sc}$  values in human colon mucosa from male and female patients.** Values in parentheses denote the number of observations.

#### 3.1.2.2 hPP responses in human colon mucosa

In contrast to the transient responses observed in Col-24 monolayers, pooled responses to hPP (100 nM) in human colon mucosa were significantly slower in the rate of onset (peaking within 10-15 min) followed by sustained reductions in  $I_{sc}$  (Figure 3.5A).

Responses to hPP in human mucosa were also concentration-dependent, where 0.1 nM - 1  $\mu\text{M}$  inhibited  $I_{sc}$ , generating an  $\text{EC}_{50}$  value of 8.8 nM (4.6-17.0 nM,  $n=3$ ) (Figure 3.5B).  $\text{Y}_4$  receptor signalling in human colon mucosa also exhibited desensitisation (Figure 3.5C), where a subsequent addition of hPP (100 nM) in preparations pre-treated with hPP (100 nM) led to a significantly smaller response, compared with that observed in naïve preparations. Responses to anti-secretory controls, PYY (100 nM) and the  $\alpha_2$ -adrenoceptor agonist, UK 14,304 (1  $\mu\text{M}$ ) were unaffected, demonstrating specificity in  $\text{Y}_4$ -activated  $\text{G}_i$  coupling. Thus, PYY and UK 14,304 were used as internal controls to assess the  $\text{Y}_2$  (and  $\text{Y}_1$ ) and  $\alpha_2$  signalling of each preparation in addition to the overall anti-secretory capability of the mucosa. A representative trace demonstrating peptide responses in human mucosa is shown in Figure 3.5D. Responses to PYY were faster in the rate of onset compared with hPP but comparable in size and were maximal by 20 min ( $-8.0 \pm 0.7 \mu\text{A} \cdot \text{cm}^{-2}$ ,  $n=122$ ). UK 14,304 consistently produced further reductions in  $I_{sc}$  ( $-21.0 \pm 1.6 \mu\text{A} \cdot \text{cm}^{-2}$ ,  $n=122$ ) indicating the potent nature of the anti-secretory agent.



**Figure 3.5 hPP responses in human colon mucosa.** (A) Pooled time course profiles of hPP (100 nM, n=3-10), added at t=0. (B) Cumulative concentration-response curve in naïve preparations (n=3). The curve provided the  $EC_{50}$  value as quoted in the text. (C) Desensitisation of the hPP-activated  $Y_4$  responses. (D) Representative responses to hPP, PYY (both at 100 nM) and the  $\alpha_2$ -agonist, UK 14,304 (1  $\mu$ M). The value to the left of the trace is the basal  $I_{sc}$  (in  $\mu A \cdot 0.64 \text{ cm}^{-2}$ ). Each bar or point represents the mean  $\pm$  S.E.M and  $n$  values are shown in parentheses. \*  $P < 0.05$  compared with control hPP ('Ctrl', 100 nM) responses measured using Student's t-test.

## 3.2 Functional assessment of Obinepitide ([Q<sup>34</sup>]hPP)

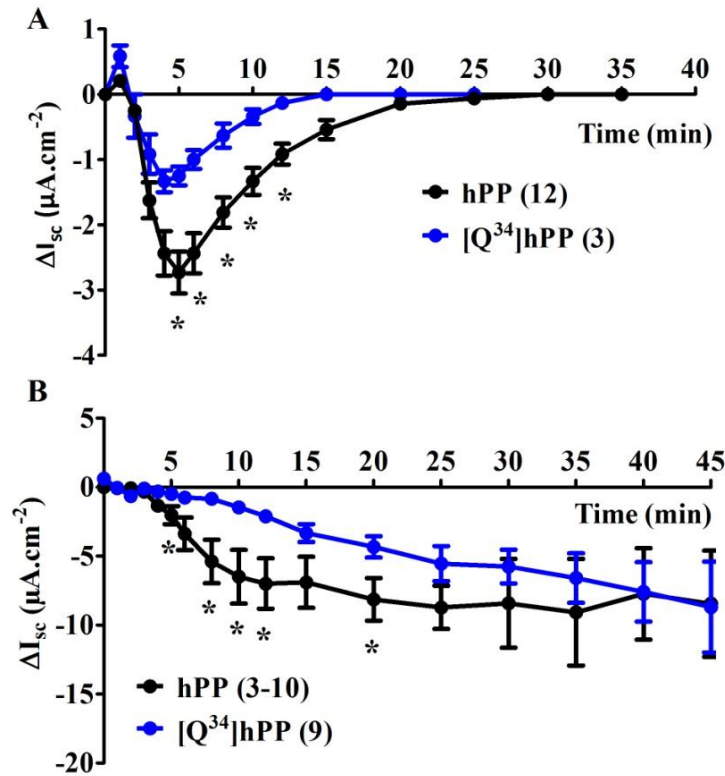
By replacing Pro<sup>34</sup> with Gln in the amino acid sequence of hPP, a dual, specific single digit nanomolar agonist at both the  $hY_2$  and the  $hY_4$  receptor was obtained, Obinepitide ([Q<sup>34</sup>]hPP). Position 34 has previously been found to be crucial in differential receptor recognition through the design of the prototype  $hY_1$  selective ligand [Pro<sup>34</sup>]NPY (Fuhlendorff *et al.*, 1990). To assess whether this modification was tolerated and whether this dual agonist peptide was able to activate both the  $Y_2$  and  $Y_4$  receptors,  $Y_4$  agonism and  $Y_2/Y_4$  agonism were characterised in Col-24 epithelial monolayers and human colon mucosa (in the absence/presence of a  $Y_2$  antagonist), respectively.

### 3.2.1 [Q<sup>34</sup>]hPP time courses and concentration-dependent effects in Col-24 epithelial monolayers and colonic mucosa

In Col-24 monolayers, the time course of a single near-maximal concentration of [Q<sup>34</sup>]hPP (100 nM) was rapid, reducing  $I_{sc}$  with a peak response within 5 min ( $-1.3 \pm 0.2$



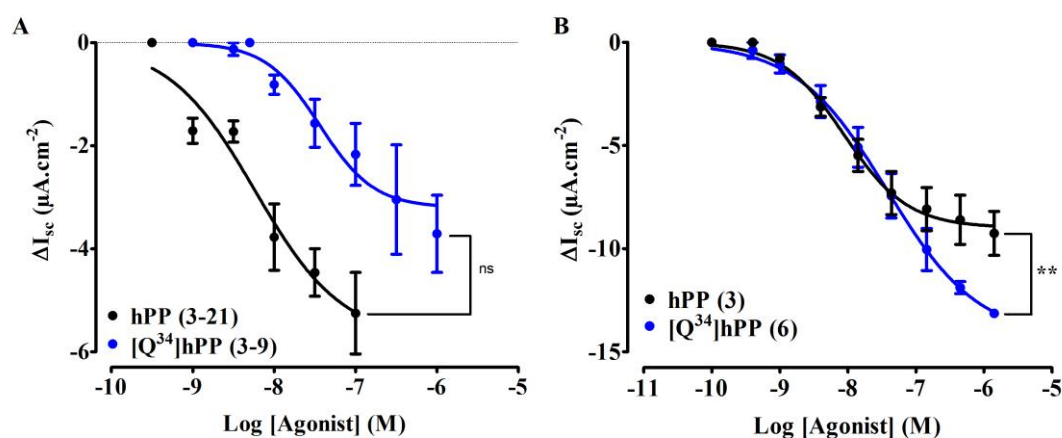
$\mu\text{A}.\text{cm}^{-2}$ ;  $n=3$ ). Although the size of the response to  $[\text{Q}^{34}]\text{hPP}$  was 50% reduced compared to that of hPP ( $-2.7 \pm 0.3 \mu\text{A}.\text{cm}^{-2}$ ;  $n=12$ ), the transient  $I_{\text{sc}}$  responses were comparable in terms of rate of onset ( $P > 0.05$  determined at each time point from 1-5 min), both returning to pre-stimulation levels within 20 min of addition (Figure 3.6A). In human colon mucosa, a 100 nM concentration of each peptide showed  $[\text{Q}^{34}]\text{hPP}$  to be significantly slower in the rate of onset compared with hPP in this  $\text{Y}_2/\text{Y}_4$  receptor-expressing preparation (Figure 3.6B).



**Figure 3.6 Comparison of the anti-secretory responses to hPP and  $[\text{Q}^{34}]\text{hPP}$ .** Time course profiles of pooled  $[\text{Q}^{34}]\text{hPP}$  responses compared with the native hormone, hPP, in (A) Col-24 monolayers and (B) human colon mucosa.  $[\text{Q}^{34}]\text{hPP}$  (100 nM in both preparations) and hPP (10 nM in Col-24 and 100 nM in human mucosa) were added basolaterally at  $t = 0$  min. Data are presented as means  $\pm$  SEM, while  $n$  numbers are indicated in parenthesis. \*  $P < 0.05$  determined using a Student's t-test.

As expected, responses to  $[\text{Q}^{34}]\text{hPP}$  were concentration-dependent in both assays. This modification resulted in a 6-fold lower potency with  $[\text{Q}^{34}]\text{hPP}$  compared with hPP in Col-24 monolayers (36.0 nM (18.0-73.0) and 5.9 nM (1.0-34.0), respectively), although statistically there was no significant difference in the efficacy between the two peptides ( $P > 0.05$ ) (Figure 3.7A). In human colon mucosa, both  $[\text{Q}^{34}]\text{hPP}$  and the native hormone exhibited similar  $\text{EC}_{50}$  to that observed in Col-24 monolayer (38.0 nM (16.0-92.0) and 8.8 nM (4.6-17.0), respectively) and in fact  $[\text{Q}^{34}]\text{hPP}$  displayed significantly increased efficacy ( $P < 0.01$ ) in this  $\text{Y}_2/\text{Y}_4$  (and  $\text{Y}_1$ ) expressing tissue (Figure 3.7B).

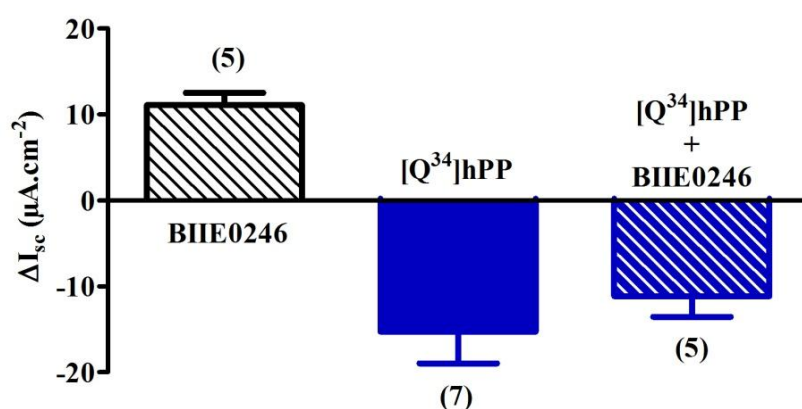




**Figure 3.7 Concentration-response profiles for [Q<sup>34</sup>]hPP compared with hPP.** (A) Non-cumulative pooled responses in Col-24 monolayers and (B) cumulative pooled responses in human colon mucosa, generating EC<sub>50</sub> values quoted in the text. Each point represents the mean  $\pm$  1 S.E.M. of pooled data. Statistical differences were determined using Student's t-test, where ns = no significant difference, \*  $P < 0.05$  and \*\*  $P < 0.01$ .

### 3.2.2 Determination of Y receptor types mediating the anti-secretory response to [Q<sup>34</sup>]hPP in human colon mucosa

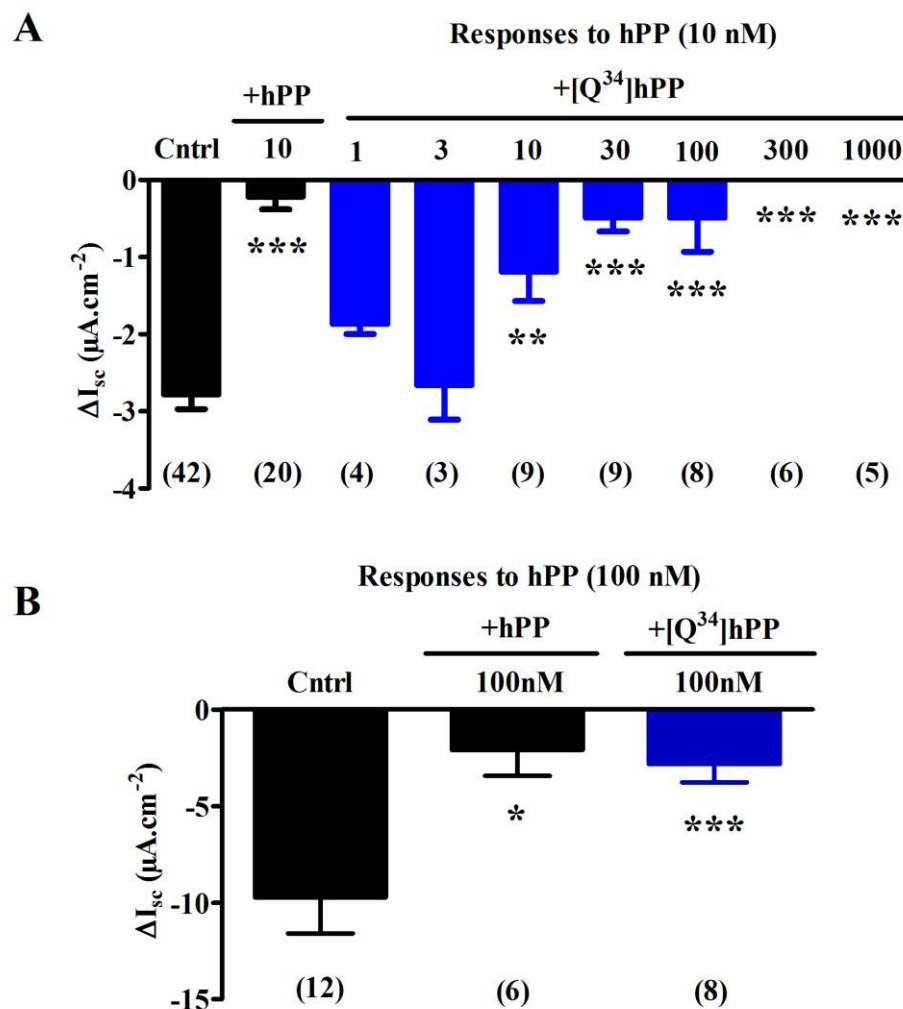
To confirm dual agonism, responses to [Q<sup>34</sup>]hPP were measured in the presence of the competitive Y<sub>2</sub> antagonist BIIE0246. [Q<sup>34</sup>]hPP (100 nM) was partially sensitive to pre-treatment with BIIE0246 (1  $\mu$ M) in human colon mucosa (Figure 3.8). The basal  $I_{sc}$  was also raised by BIIE0246, revealing tonic Y<sub>2</sub> activity, as previously reported (Cox and Tough 2002, Hyland *et al.* 2003). The size of the response to [Q<sup>34</sup>]hPP in the presence of BIIE0246 was reduced but not significantly so compared to [Q<sup>34</sup>]hPP alone (Figure 3.8), confirming the ability of this analogue to activate Y<sub>4</sub> and (to a lesser degree) Y<sub>2</sub> receptors in these preparations.



**Figure 3.8 The effect of Y<sub>2</sub> antagonism on the anti-secretory response to [Q<sup>34</sup>]hPP in human colon mucosa.** The Y<sub>2</sub> antagonist BIIE0246 alone raised  $I_{sc}$  and anti-secretory responses to [Q<sup>34</sup>]hPP (100 nM) in the presence of BIIE0246 (1  $\mu$ M) were partially inhibited. Each bar represents the mean  $\pm$  1 S.E.M. of pooled responses and the  $n$  numbers are shown in parenthesis.

### 3.2.3 Desensitisation of hPP responses in Col-24 monolayers with [Q<sup>34</sup>]hPP

Responses to hPP (10 nM) added 25 min after pre-treatment with increasing concentrations of [Q<sup>34</sup>]hPP (100 nM) elicited concentration-dependent reductions in the size of the second hPP response (Figure 3.9), comparable to that of the native hormone (see Figure 3.4). The desensitisation of hPP-activated responses were significant at concentrations of 10 nM and higher of [Q<sup>34</sup>]hPP ( $P < 0.01$ , Figure 3.9A). A significant degree of Y<sub>4</sub> desensitisation was also observed in human colon mucosa, where pre-treatment with 100 nM [Q<sup>34</sup>]hPP caused significant reductions in the response size to hPP (100 nM; Figure 3.9B).



**Figure 3.9 Desensitisation of the hPP Y<sub>4</sub> responses after activation by [Q<sup>34</sup>]hPP.** (A) Responses to hPP (10 nM) added 25 min after pre-treatment with increasing concentration of [Q<sup>34</sup>]hPP (1 nM-1 μM) in Col-24 monolayers. (B) Responses to hPP (100 nM) added 60 min after pre-treatment with [Q<sup>34</sup>]hPP (100 nM) in human colon mucosa. Each bar represents the mean  $\pm$  1 S.E.M and *n* values are shown in parentheses. Significant differences are shown, where \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , compared with control hPP ('Cntrl', 10nM) responses, measured using a one-way ANOVA and Dunnett's post-test.

### 3.3 Functional assessment of PEG- and fatty acid- conjugated [K<sup>30</sup>, Q<sup>34</sup>]hPP analogues

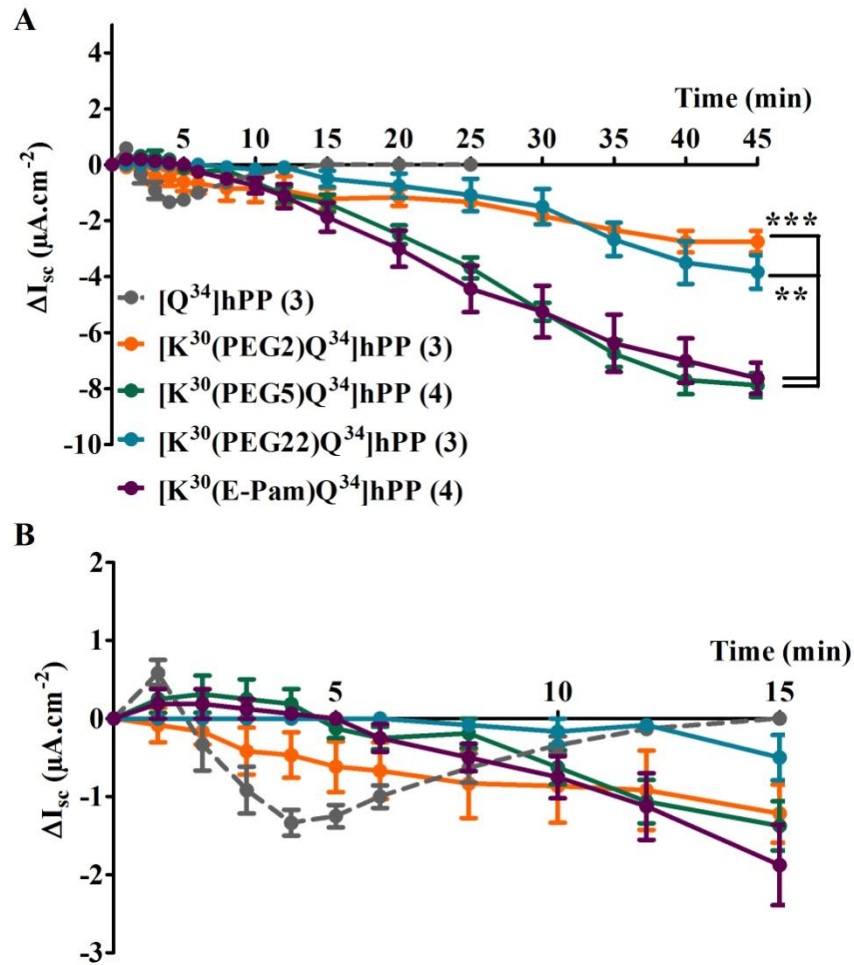
To elongate the actions of [Q<sup>34</sup>]hPP, a Lys at position 30 was introduced by our collaborators that enabled site-specific modification with either a 2, 5 or 22 kDa PEG moiety (referred to as PEG2, PEG5 and PEG22) or a palmitoyl (Pam) moiety. The introduction of Lys<sup>30</sup> resulted in retention of the binding capacity and demonstrated nanomolar affinities for both the hY<sub>2</sub> and hY<sub>4</sub> receptor, as demonstrated by competitive binding assays in which <sup>125</sup>I-labeled binding ligands were incubated at a constant concentration together with the analogues in varying concentrations on COS7 cells transfected with the receptor to be investigated (Bellmann-Sickert *et al*, 2011). Due to the lack of chemical stability with [K<sup>30</sup>, Q<sup>34</sup>]hPP, this peptide was unavailable for assessment. The resulting PEG-modified peptides, [K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP and [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP, exhibited a marginal decrease in IP<sub>3</sub> accumulation subsequent to hY<sub>4</sub> activation (Beck-Sickinger *et al*. Appendix 1.0, Table 1) compared to the unmodified ligand. Modification of [K<sup>30</sup>, Q<sup>34</sup>]hPP with palmitic acid resulted in a retention of potency in the case of the hY<sub>2</sub> and hY<sub>4</sub> receptor (Beck-Sickinger *et al*. Appendix 1.0, Table 1).

#### 3.3.1 Time course profiles of epithelial and mucosal I<sub>sc</sub> responses with [K<sup>30</sup>, Q<sup>34</sup>]hPP analogues

The impact of increasing PEG polymer size and fatty acid modification on longevity of action was investigated and compared with [Q<sup>34</sup>]hPP responses. In Col-24 monolayers, Y<sub>4</sub> agonism with [K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP, [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP and [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP (100 nM) resulted in reductions in I<sub>sc</sub> that were prolonged, where maxima was not reached until 45 min (Figure 3.10A). Although the effect of a PEG2 modification resulted in a sustained anti-secretory response, there was no significant difference in the maxima compared with [Q<sup>34</sup>]hPP (at 4 min versus 45 min with PEG2-peptide), albeit at a later time point. On the other hand, modification with PEG5 was well tolerated and resulted in a much greater response size; maximum reductions in I<sub>sc</sub> were significantly greater (at 45 min) compared with responses to [K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP ( $P < 0.001$ ) at the same time point. Conversely, modification with a PEG22 polymer was detrimental to the peptide's ability to reduce I<sub>sc</sub> and resulted in a significant loss in biological activity (at 45 min,  $P < 0.01$ ), with the maximum peak response being more akin to that of [K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP. The C16 lipid-conjugated peptide, [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP, was also

well tolerated, with an anti-secretory profile comparable to that seen with  $[K^{30}(PEG5)Q^{34}]hPP$ .

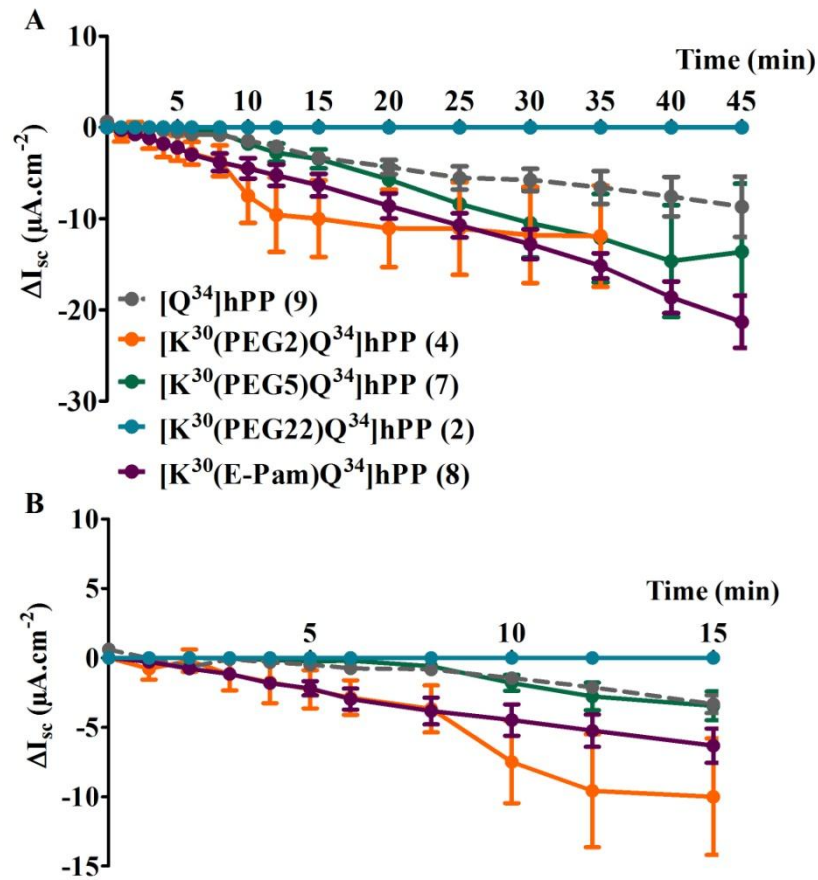
Interestingly, each PEG-modified peptide exhibited a delay in the rate of onset; an effect that was more pronounced with increasing PEG size.  $[K^{30}(E-Pam)Q^{34}]hPP$  also demonstrated this slow onset, and again this response was analogous to that observed with  $[K^{30}(PEG5)Q^{34}]hPP$  (Figure 3.10B).



**Figure 3.10 Anti-secretory responses to chemically modified  $[K^{30}, Q^{34}]hPP$  variants in Col-24 monolayers.** (A) Reductions in  $I_{sc}$  in response to a near maximal concentration (all at 100 nM) of  $[K^{30}(PEG2)Q^{34}]hPP$ ,  $[K^{30}(PEG5)Q^{34}]hPP$ ,  $[K^{30}(PEG22)Q^{34}]hPP$  and  $[K^{30}(E-Pam)Q^{34}]hPP$  are shown for a period of 45 min. (B) A magnification of early time points between 0 and 15 min for each modified peptide. Data are presented as the means  $\pm$  1 SEM, while  $n$  numbers are indicated in parenthesis. Multiple comparisons were made using one-way ANOVA with Bonferroni's post-test of the peak response size, \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared with each analogue.

In human colon mucosa single basolateral additions of the same agonists (100 nM) also revealed protracted activities (Figure 3.11A) with an indication that the response to  $[K^{30}(PEG2)Q^{34}]hPP$  reached its maximum within 12-15 min. Again,

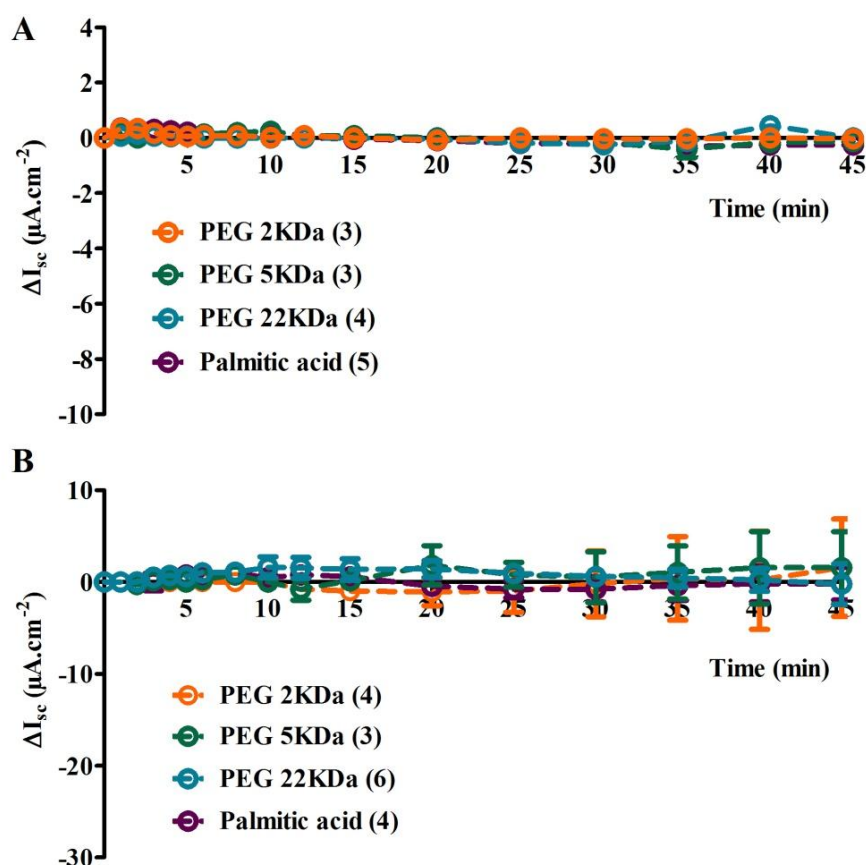
$[K^{30}(PEG5)Q^{34}]hPP$  and  $[K^{30}(E-Pam)Q^{34}]hPP$  were comparable in their time course profiles, with similar maximal responses generated (however there were no statistically significant differences compared with the that of  $[Q^{34}]hPP$ ). Additionally, each peptide was slower in onset, with  $[K^{30}(PEG2)Q^{34}]hPP$  and  $[K^{30}(E-Pam)Q^{34}]hPP$  being more rapid (Figure 3.11B). Interestingly in the isolated mucosa preparations,  $[K^{30}(PEG22)Q^{34}]hPP$  (100 nM) displayed a complete loss of anti-secretory activity, with no reduction in  $I_{sc}$  being observed within the 45 min observation time. Increasing the concentration to 3  $\mu M$   $[K^{30}(PEG22)Q^{34}]hPP$  still demonstrated the absence of biological activity (data not shown).



**Figure 3.11 Anti-secretory responses to chemically modified  $[K^{30}, Q^{34}]hPP$  variants in human colon mucosa.** In (A) reductions in  $I_{sc}$  in response to 100 nM  $[K^{30}(PEG2)Q^{34}]hPP$ ,  $[K^{30}(PEG5)Q^{34}]hPP$ ,  $[K^{30}(PEG22)Q^{34}]hPP$  and  $[K^{30}(E-Pam)Q^{34}]hPP$  are shown for a period of 45 min. (B) A magnification of early time points between 0 and 15 min for each modified peptide. Data are presented as the means  $\pm$  1 SEM, while  $n$  numbers are indicated in parenthesis. Multiple comparisons were made using one-way ANOVA with Bonferroni's post-test and there were no significant differences.

### 3.3.2 PEG polymer and palmitic acid responses in Col-24 epithelial and colonic mucosal preparations

To exclude the possibility that the anti-secretory responses observed with the chemically modified  $[K^{30}, Q^{34}]hPP$  variants were due to the PEG polymers and palmitic acid side chains *per se*, additions of PEG2, PEG5, PEG22 and palmitic acid (all at 100 nM) were made to both Col-24 monolayers (Figure 3.12A) and colon mucosa preparations (Figure 3.12B). Within the observation time of 45 min, all side chains had no effect upon  $I_{sc}$  levels in either preparation.



**Figure 3.12 Lack of activity with PEG2, PEG5, PEG22 and palmitic acid.** No changes in  $I_{sc}$  were seen following additions of each side chain (100 nM) in (A) Col-24 monolayers and (B) human colon mucosa. Data are presented as the means  $\pm$  1 SEM, while  $n$  numbers are indicated in parenthesis.

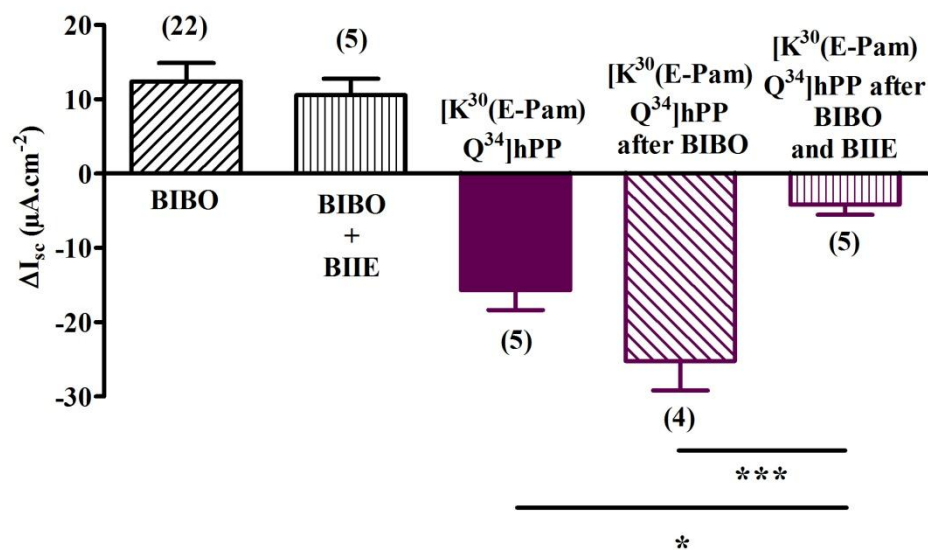
### 3.3.3 Effects of a selective $Y_1$ antagonist upon $[K^{30}(E-Pam)Q^{34}]hPP$ anti-secretory responses in human colonic mucosal specimens

In binding studies performed by our collaborators (Beck-Sickinger *et al.*, see Appendix 1.0, Table 1),  $[K^{30}(E-Pam)Q^{34}]hPP$  exhibited nM potency for the  $hY_1$  receptor (albeit two orders of magnitude lower than  $Y_4$  and  $Y_2$  activities). To ascertain whether  $Y_1$  activation occurred in isolated human colonic specimens (known to express  $Y_1$  as well as  $Y_4$  and  $Y_2$  receptors; Tough and Cox, 2002; Hyland *et al.*, 2003; Tough *et al.*, 2006),



tissues were pre-treated with an optimal concentration of the  $Y_1$  antagonist, BIBO3304 (300 nM), which alone raised  $I_{sc}$  levels from the basal level of secretion, indicating tonic  $Y_1$  activity (as observed previously, Tough and Cox, 2002). Although  $I_{sc}$  levels were raised in tissues following BIBO3304 ( $71.9 \pm 22.3 \mu A.cm^{-2}$ ,  $n=5$ ) these levels of secretion prior to the addition of  $[K^{30}(E-Pam)Q^{34}]hPP$  did not alter significantly to those observed in tissues exposed to  $[K^{30}(E-Pam)Q^{34}]hPP$  only ( $87.7 \pm 17.5 \mu A.cm^{-2}$ ,  $n=5$ ).

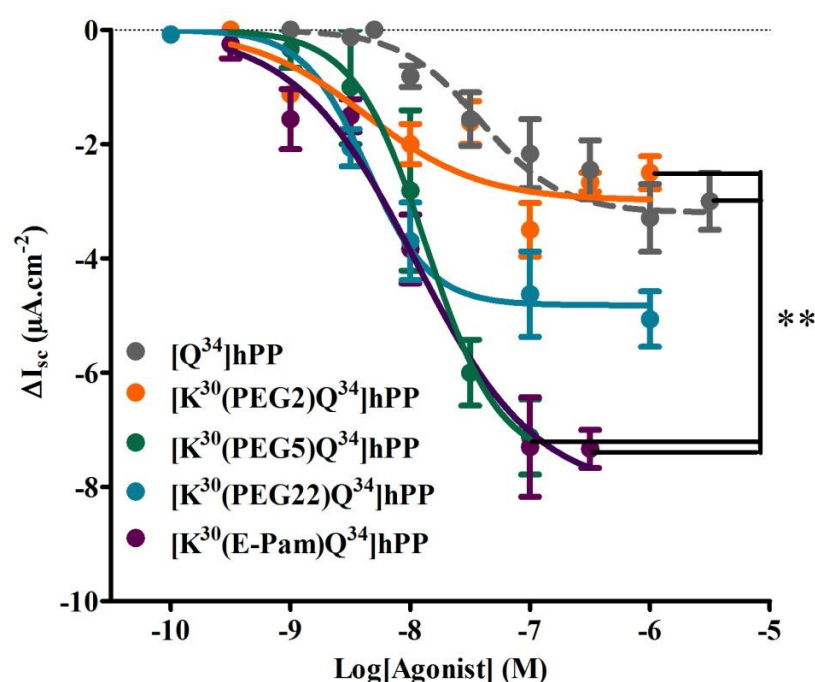
In the presence of BIBO3304, responses to  $[K^{30}(E-Pam)Q^{34}]hPP$  (100 nM) were not significantly reduced (Figure 3.13). In fact, the size of the response was larger (but not significantly so). In the presence of both BIIE0246 (1  $\mu M$ ) and BIBO3304 (300 nM), the anti-secretory response to  $[K^{30}(E-Pam)Q^{34}]hPP$  was partially inhibited ( $P < 0.05$ ) compared to the response observed for  $[K^{30}(E-Pam)Q^{34}]hPP$  in naive preparations, indicating the significance of the  $Y_2$  receptor in the anti-secretory response to  $[K^{30}(E-Pam)Q^{34}]hPP$ . It should be noted however that pre-existing  $I_{sc}$  level following dual addition of BIIE0246 and BIBO3304 ( $86.4 \pm 13.0$ ) was not statistically significant compared to that seen in naive tissues where no antagonist was added.



**Figure 3.13 The effects of  $Y_1$  and  $Y_2$  antagonism on the anti-secretory responses to  $[K^{30}(E-Pam)Q^{34}]hPP$  in human colon mucosa.** Increases in  $I_{sc}$  were observed in response to BIBO3304 (300 nM) and BIIE0246 (1  $\mu M$ ) in naive preparations, demonstrating  $Y_1$  and  $Y_2$  mediated tone. Responses to  $[K^{30}(E-Pam)Q^{34}]hPP$  (100 nM) are compared in the absence/presence of antagonists as shown, and statistical differences were determined using a Student's t-test, where \*  $P < 0.05$ , \*\*\*  $P < 0.001$ . Each bar represents the mean  $\pm$  1 S.E.M. of pooled responses with  $n$  numbers shown in parenthesis.

### 3.3.4 Concentration-response relationships between $[K^{30}, Q^{34}]hPP$ modified analogues

In Col-24 monolayers, concentration-response curves indicated that chemical modifications were well tolerated with no apparent detriment to agonist potency. In fact, all chemically modified peptides were an order of magnitude more potent compared with their unmodified peptide,  $[Q^{34}]hPP$  (Figure 3.14). The resulting  $EC_{50}$  values are shown in Table 3.3. A one way ANOVA and Bonferroni's post-test revealed significantly greater efficacy with  $[K^{30}(PEG5)Q^{34}]hPP$  and  $[K^{30}(E-Pam)Q^{34}]hPP$  compared with  $[K^{30}(PEG2)Q^{34}]hPP$  and their non-modified predecessor ( $P < 0.01$ ).



**Figure 3.14 Peptide responses in Col-24 monolayers.** Non-cumulative concentration-response profiles for  $[K^{30}(PEG2)Q^{34}]hPP$  ( $n=3-6$ ),  $[K^{30}(PEG5)Q^{34}]hPP$  ( $n=3-8$ ),  $[K^{30}(PEG22)Q^{34}]hPP$  ( $n=3-4$ ) and  $[K^{30}(E-Pam)Q^{34}]hPP$  ( $n=3-5$ ) compared with  $[Q^{34}]hPP$  ( $n=3-9$ ). The peak responses for PEGylated and lipidated peptides were measured at approx. 45 min compared with  $[Q^{34}]hPP$  at 5 min (grey dashed line), generating  $EC_{50}$  values and 95% confidence limits quoted in Table 3.3. Each point represents the mean  $\pm$  1 S.E.M of pooled data. Statistical differences were determined using a one way ANOVA with Bonferroni's post-test, where \*\*  $P < 0.01$ .



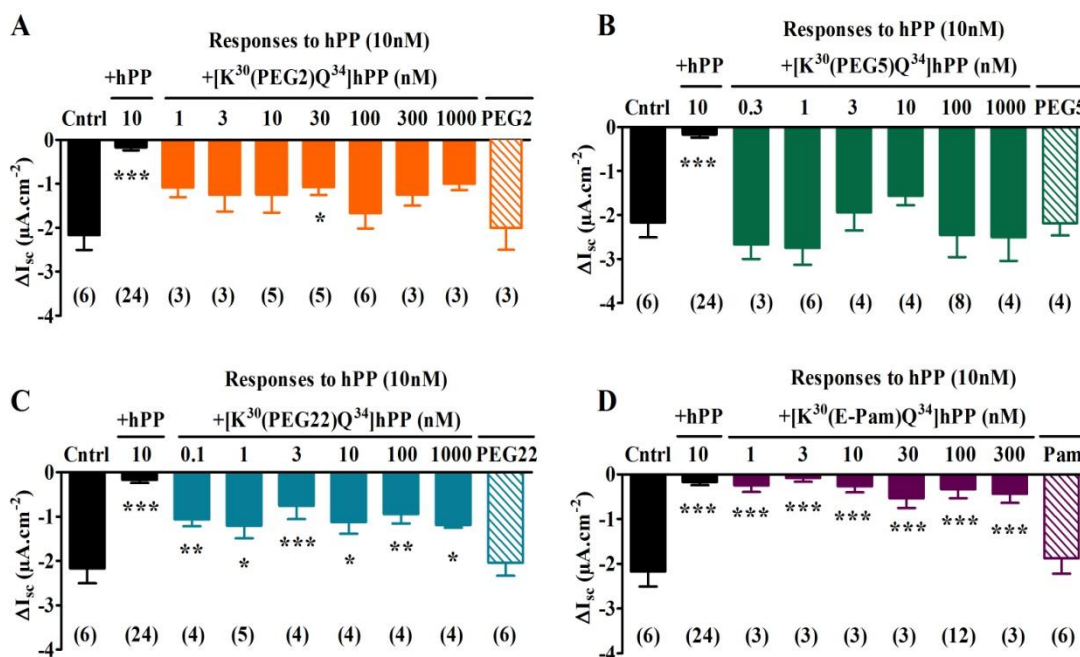
Peptide	EC <sub>50</sub> value (nM)	95% confidence
[Q <sup>34</sup> ]hPP	36.0	18.0-73.0
[K <sup>30</sup> (PEG2)Q <sup>34</sup> ]hPP	4.3	1.0-18.0
[K <sup>30</sup> (PEG5)Q <sup>34</sup> ]hPP	13.8	5.9-32.0
[K <sup>30</sup> (PEG22)Q <sup>34</sup> ]hPP	4.3	2.6-7.2
[K <sup>30</sup> (E-Pam)Q <sup>34</sup> ]hPP	11.1	3.4-36.0

**Table 3.3 Comparison of chemically modified [K<sup>30</sup>, Q<sup>34</sup>]hPP EC<sub>50</sub> values (with 95% confidence limits).** Data was calculated from non-cumulative concentration-response curves obtained from Col-24 monolayers.

### 3.3.5 Desensitisation of hPP-activated Y<sub>4</sub> responses with [K<sup>30</sup>, Q<sup>34</sup>]hPP chemically modified analogues

The consequence of PEG and fatty acid modification of [K<sup>30</sup>, Q<sup>34</sup>]hPP on subsequent Y<sub>4</sub> receptor desensitisation was assessed next. The measurement of hPP (10 nM) responses following pre-treatment with a PEGylated [K<sup>30</sup>, Q<sup>34</sup>]hPP variant (100 nM) revealed unexpected differences that indicated an apparent lack of desensitisation specifically with PEG5 and perhaps the PEG2 analogue (Figure 3.15) not seen with any other non-modified peptides. Responses to hPP added 45 min after pre-treatment with a range of [K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP and [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP concentrations were unaltered compared to control hPP responses, (with the exception of 30 nM [K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP (Figure 3.15A) and despite potent Y<sub>4</sub> activation with this PEGylated peptide (Figure 3.14). In contrast [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP (0.1 nM- 1 µM) additions resulted in partial inhibition of subsequent hPP responses, which were significantly reduced compared to hPP ‘Cntrl’ values in a concentration-independent manner (Figure 3.15C).

On the contrary, significant desensitisation to low nM concentrations of [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP was observed (Figure 3.15D). Pre-incubation with [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP (1-300 nM) significantly attenuated further Y<sub>4</sub> signalling to subsequent hPP responses ( $P < 0.01$ ). Importantly, the different PEG polymers or palmitic acid (100 nM) side chains alone had no effect upon Y<sub>4</sub> desensitisation (Figure 3.15) indicating that the differential sizes of the subsequent hPP response were a peptide-specific effect. Thus it appears that the lipidated PP analogue is internalised rapidly while PEGylated peptides do not cause significant Y<sub>4</sub> receptor desensitisation at the same low concentration once bound to the Y<sub>4</sub> receptor. This divergence was also observed in imaging studies performed by our collaborators (Discussed in Chapter 4), where Y<sub>4</sub> receptor internalisation was determined with the use of a yellow fluorescent protein (YFP)-tagged Y<sub>4</sub> receptor.

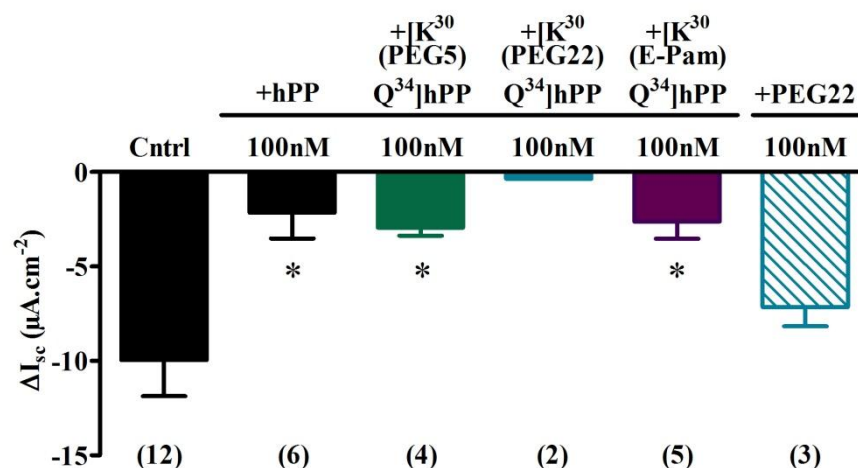


**Figure 3.15 Desensitisation of hPP-activated responses following pre-treatment with chemically modified [K<sup>30</sup>, Q<sup>34</sup>]hPP variants in Col-24 monolayers.**

Desensitisation of hPP responses (10 nM throughout) added 45 min after increasing concentrations of either (A) [K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP, (B) [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP, (C) [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP or (D) [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP, showing differential desensitisation of subsequent hPP responses. Responses to hPP following different PEG or palmitic acid side chains are shown to the right of each histogram (hatched bars). Each bar represents the mean  $\pm$  1 S.E.M with *n* numbers shown in parenthesis. Statistical differences from 'Cntrl' hPP responses were determined using ANOVA and Dunnett's post-test, where \* *P* < 0.05, \*\* *P* < 0.01 and \*\*\* *P* < 0.001.

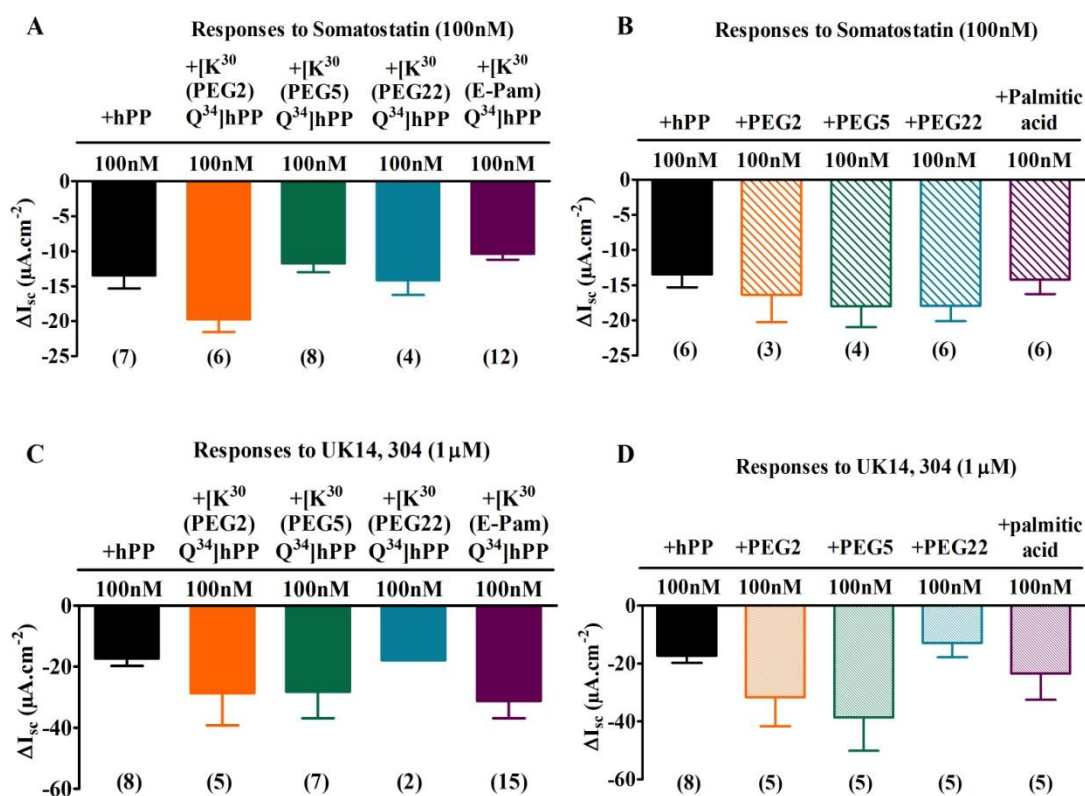
Given the divergence in Y<sub>4</sub> receptor desensitisation between the PEGylated (specifically PEG5) and palmitoylated peptide observed in Col-24 layers, desensitisation of hPP responses following pre-treatment with a single concentration of [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP, [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP or [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP was assessed in isolated colon mucosa (Figure 3.16). The limited access to human tissue precluded assessment of a range of modified agonist concentrations upon hPP desensitisation. Despite this, human colon mucosal pre-treatment with [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP (100 nM) resulted in a significant reduction in the subsequent hPP responses (73.1 %  $\pm$  0.9, *n*=5) compared with 'Cntrl' hPP (100 nM), mimicking the loss of Y<sub>4</sub> responses observed in Col-24 monolayers. Unexpectedly, [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP (100 nM) also significantly reduced the subsequent hPP response by 71.0 %  $\pm$  0.4 (*n*=4), contrary to the lack of desensitisation observed in Col-24 monolayers. In agreement with that seen in Col-24 monolayers, [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP desensitised the Y<sub>4</sub> receptor and unexpectedly, the loss in Y<sub>4</sub> signal was greater than that induced by [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP (96.0 %), albeit with 2

observations. Importantly, this loss in  $Y_4$  signalling was not a direct result of the PEG polymer, since PEG22 (100 nM) had no significant effect upon the  $Y_4$ -mediated hPP response size (Figure 3.16).



**Figure 3.16 Desensitisation of hPP activated responses following pre-treatment with chemically modified analogues in human colon mucosa.** Desensitisation of hPP responses (100 nM throughout) added 60 min after the addition of  $[K^{30}(PEG5)Q^{34}]hPP$ ,  $[K^{30}(PEG22)Q^{34}]hPP$  or  $[K^{30}(E-Pam)Q^{34}]hPP$ , and after PEG22 polymer (100 nM, hatched bar). Each bar represents the mean  $\pm$  1 S.E.M with  $n$  numbers shown in parenthesis. Statistical differences from ‘Cntrl’ hPP responses were determined using ANOVA and Dunnett’s post-test, where \*  $P < 0.05$ .

Although a considerable level of  $Y_4$  desensitisation was evident in these tissues, each  $[K^{30}, Q^{34}]hPP$  variant and their respective PEG/lipid conjugates did not affect other  $G_{ai}$ -coupled anti-secretory responses; 100 nM somatostatin responses were unaltered (Figure 3.17A&B) in Col-24 monolayers, as well as 1  $\mu$ M UK 14,304 responses (Figure 3.17C&D) in human colon mucosa, suggesting  $Y_4$  receptor specificity of the PEG/lipid-modified dual  $Y_2/Y_4$  agonists.



**Figure 3.17 Responses to somatostatin (100 nM) and UK 14,304 (1  $\mu$ M) in Col-24 monolayers and human colon mucosa, respectively, following pre-treatment with PEG/lipid-modified  $[K^{30}, Q^{34}]hPP$  variants.** Selectivity of  $G_{ai}$ - coupled signalling demonstrated by insignificant changes in the response to somatostatin (100 nM) after pre-treatment with (A)  $[K^{30}(PEG2)Q^{34}]hPP$ ,  $[K^{30}(PEG5)Q^{34}]hPP$ ,  $[K^{30}(PEG22)Q^{34}]hPP$  and  $[K^{30}(E-Pam)Q^{34}]hPP$  and (B) their respective PEG polymer or fatty acid (all at 100 nM) in Col-24 monolayers. Responses to UK 14,304 (1  $\mu$ M) in human colon mucosa are shown in (C) and (D). Each bar represents the mean  $\pm$  1 S.E.M with *n* numbers shown in parenthesis. There were no statistical differences from control hPP responses as determined by ANOVA with Dunnett's post-test.

### 3.4 Functional assessment of PEG- and fatty acid- conjugated $[K^{22}, Q^{34}]hPP$ analogues

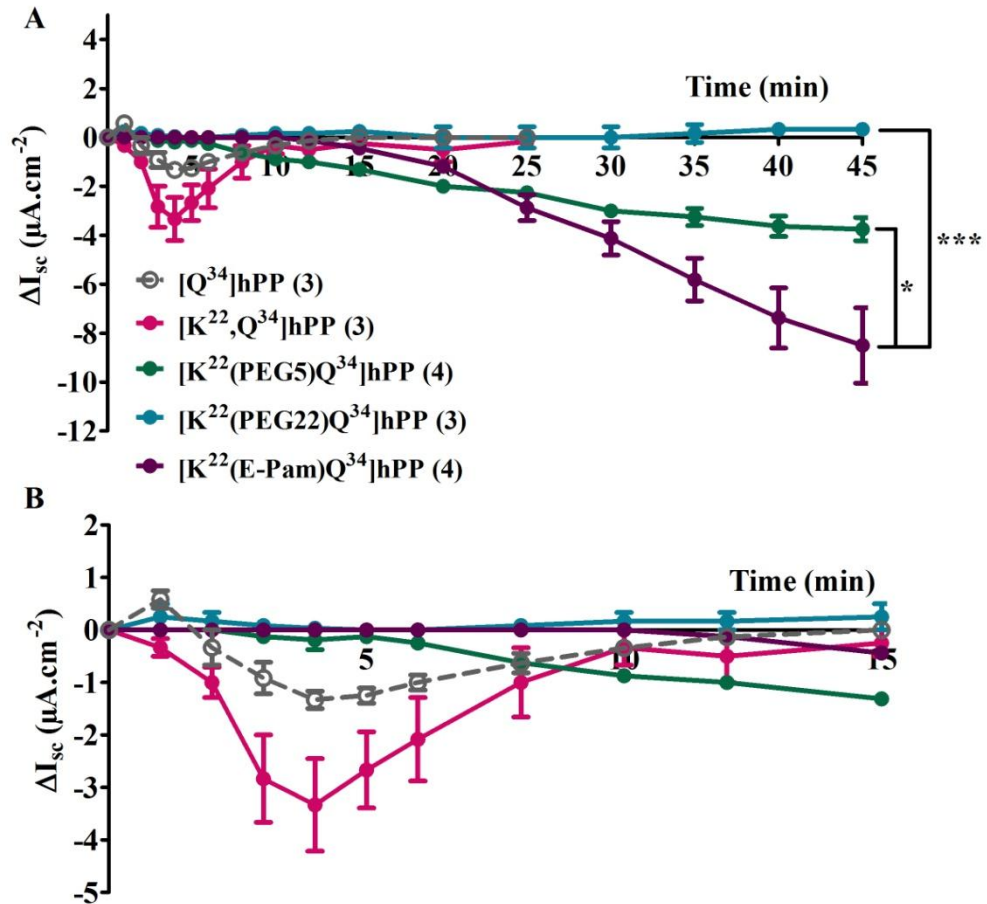
A lysine substitution at position 30 of the PP sequence has proven to be a successful linker for PEG polymer conjugation with respect to functional  $Y_4$  anti-secretory responses in both colonic specimens. Initial studies performed by our collaborators have revealed a 20-fold longer half life with a PEG22ylated  $K^{22}$  variant compared with its non-PEGylated analogues,  $[K^{22}, Q^{34}]hPP$ , determined by incubation of both peptides (modified with fluorophore 5(6)-carboxyfluorescein) with porcine liver extract homogenates (as a measure of protease resistance) and *in vitro* bioavailability (unpublished data, Made *et al.*). Lysine modification at this position also led to nanomolar activity at both the  $Y_2$  and  $Y_4$  receptor with only a small loss in potency with increasing PEG size, in addition to selectivity over the  $Y_1$  and  $Y_5$  receptor, as

determined by an IP<sub>3</sub> turnover assay (see Appendix 1.0 for Y receptor potencies). Since these analogues displayed promising *in vitro* properties, it was important to test their *in vitro* pharmacology and compare it with their [K<sup>30</sup>, Q<sup>34</sup>]hPP equivalents..

### 3.4.1 Time course profiles of epithelial and mucosal I<sub>sc</sub> responses with [K<sup>22</sup>, Q<sup>34</sup>]hPP analogues

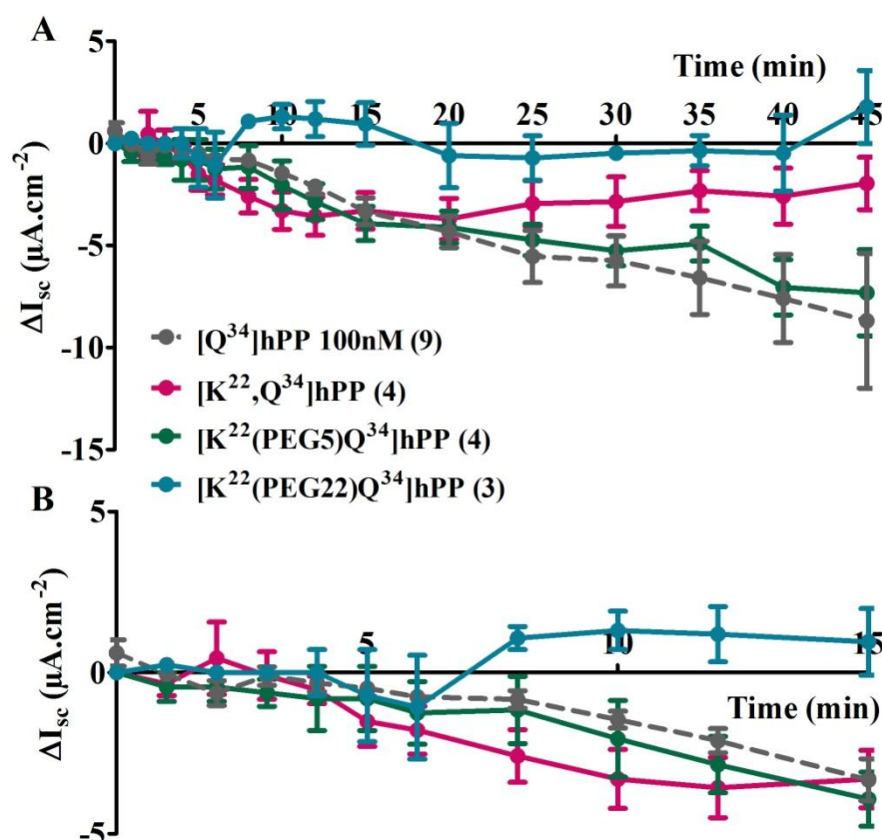
In Col-24 monolayers the non-modified peptide, [K<sup>22</sup>, Q<sup>34</sup>]hPP, exhibited a comparable time course profile to hPP and [Q<sup>34</sup>]hPP, where a rapid rate of onset and maxima at approximately 5 min was observed with 100 nM (Figure 3.18). The presence of a PEG5 polymer slowed the rate of onset considerably, as seen with [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP, followed by prolonged reductions in I<sub>sc</sub> with an indication of maxima being reached at 45 min. Interestingly, the presence of a PEG22 polymer led to complete inactivity within the observation period of 45 min. Palmitoylation of [K<sup>22</sup>, Q<sup>34</sup>]hPP also displayed the slow rate of onset seen with [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP; onset did not occur until 8 min post peptide addition, compared to 5 min observed with [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP. Despite the difference in the rate of onset between the two palmitoylated peptides, overall maxima were comparable.

The trends in the time course profiles observed in Col-24 monolayers were reproduced in human colon mucosa (Figure 3.19). Responses to [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP were prolonged compared to those of [K<sup>22</sup>, Q<sup>34</sup>]hPP, which appeared to reach maxima at approximately 20 min. As seen with [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP, [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP was inactive, however it must be noted that there were no significant differences in the maximum response size generated between all peptides.



**Figure 3.18 Anti-secretory responses to chemically modified [K<sup>22</sup>, Q<sup>34</sup>]hPP variants in Col-24 monolayers.** (A) Reductions in  $I_{sc}$  in response to a near maximal concentration (all at 100 nM) of [Q<sup>34</sup>]hPP, [K<sup>22</sup>, Q<sup>34</sup>]hPP, [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP, [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP and [K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP are shown for a period of 45 min. (B) A magnification of early time points between 0 and 15 min for each peptide. Data are presented as the means  $\pm$  1 SEM, while  $n$  numbers are indicated in parenthesis. Multiple comparisons were made using one-way ANOVA with Bonferroni's post-test of the peak response size, \*  $P < 0.05$ , \*\*\*  $P < 0.001$  compared with each analogue.



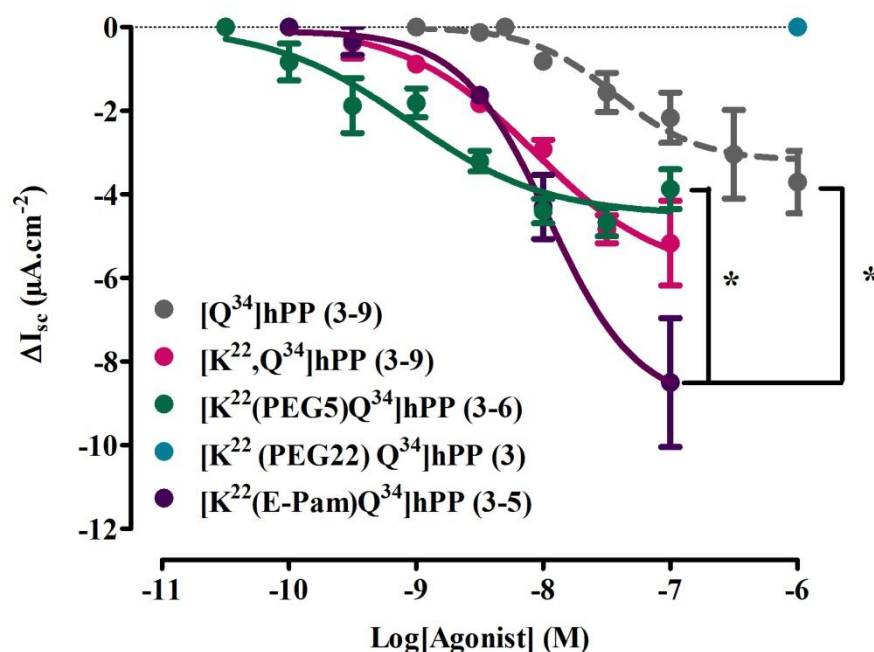


**Figure 3.19 Anti-secretory responses to chemically modified [K<sup>22</sup>, Q<sup>34</sup>]hPP variants in human colon mucosa.** (A) Reductions in  $I_{sc}$  in response to 100 nM [Q<sup>34</sup>]hPP, [K<sup>22</sup>, Q<sup>34</sup>]hPP, [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP and [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP are shown for a period of 45 min. (B) A magnification of early time points between 0 and 15 min for each peptide. Data are presented as the means  $\pm$  1 SEM, while  $n$  numbers are indicated in parenthesis. Multiple comparisons were made using one-way ANOVA with Bonferroni's post-test and there were no significant differences in the maxima.

### 3.4.2 Concentration-response relationships between [K<sup>22</sup>, Q<sup>34</sup>]hPP modified analogues

Next the [K<sup>22</sup>, Q<sup>34</sup>]hPP variants and their PEG-/lipid- derivatives were assessed for their Y<sub>4</sub> potency in Col-24 monolayers. A K<sup>22</sup> modification of [Q<sup>34</sup>]hPP resulted in a peptide which was 3 times more potent than [Q<sup>34</sup>]hPP itself, as determined by concentration-response relationships (Figure 3.20) and subsequent EC<sub>50</sub> values (Table 3.4). As seen with the [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP, PEGylation of [K<sup>22</sup>, Q<sup>34</sup>]hPP with a PEG5 polymer was not detrimental to agonist potency, in fact [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP was an order of magnitude more potent than [K<sup>22</sup>, Q<sup>34</sup>]hPP. The response maxima used to determine the potencies however were achieved at different time points (approximately 5 min for the transient response to [K<sup>22</sup>, Q<sup>34</sup>]hPP and 45 min for the longer acting [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP). Although [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP demonstrated increased potency compared with its non-PEGylated equivalent, the efficacy did not significantly differ

between the two peptides, despite the prolonged reductions in  $I_{sc}$  observed with  $[K^{22}(PEG5)Q^{34}]hPP$  (Figure 3.18). Unexpectedly,  $[K^{22}(PEG22)Q^{34}]hPP$  was inactive across the concentration range used (0.3-1  $\mu M$ , only 1  $\mu M$  is shown in Figure 3.20). The consequence of palmitoylation was significantly increased efficacy compared with  $[Q^{34}]hPP$ , and this was analogous to the responses observed with  $[K^{30}(E-Pam)Q^{34}]hPP$ .  $Y_4$  potency for  $[K^{22}(E-Pam)Q^{34}]hPP$  was also equivalent to  $[K^{22}, Q^{34}]hPP$  and hence 3 times more potent than  $[Q^{34}]hPP$ . Interestingly,  $[K^{22}(E-Pam)Q^{34}]hPP$  also displayed a significantly increased efficacy compared with the PEG5 equivalent and this was in contrast to the equi-effective responses seen with the palmitoylated and PEG5-conjugated  $[K^{30}, Q^{34}]hPP$  series of peptides.



**Figure 3.20 Peptide responses in Col-24 monolayers.** Non-cumulative concentration-response profiles for  $[K^{22}, Q^{34}]hPP$ ,  $[K^{22}(PEG5)Q^{34}]hPP$  and  $[K^{22}(E-Pam)Q^{34}]hPP$  compared with  $[Q^{34}]hPP$ . The response to a single concentration of 1  $\mu M$   $[K^{22}(PEG22)Q^{34}]hPP$  is shown. The peak responses for PEGylated and lipidated peptides were measured at ~45 min compared with  $[Q^{34}]hPP$  and  $[K^{22}, Q^{34}]hPP$  at 5 min, generating  $EC_{50}$  values and 95% confidence limits quoted in Table 3.4. Each point represents the mean  $\pm$  1 S.E.M of pooled data, with  $n$  numbers shown in parenthesis. Statistical differences were determined using a one way ANOVA with Bonferroni's post-test, where \*  $P < 0.05$ .



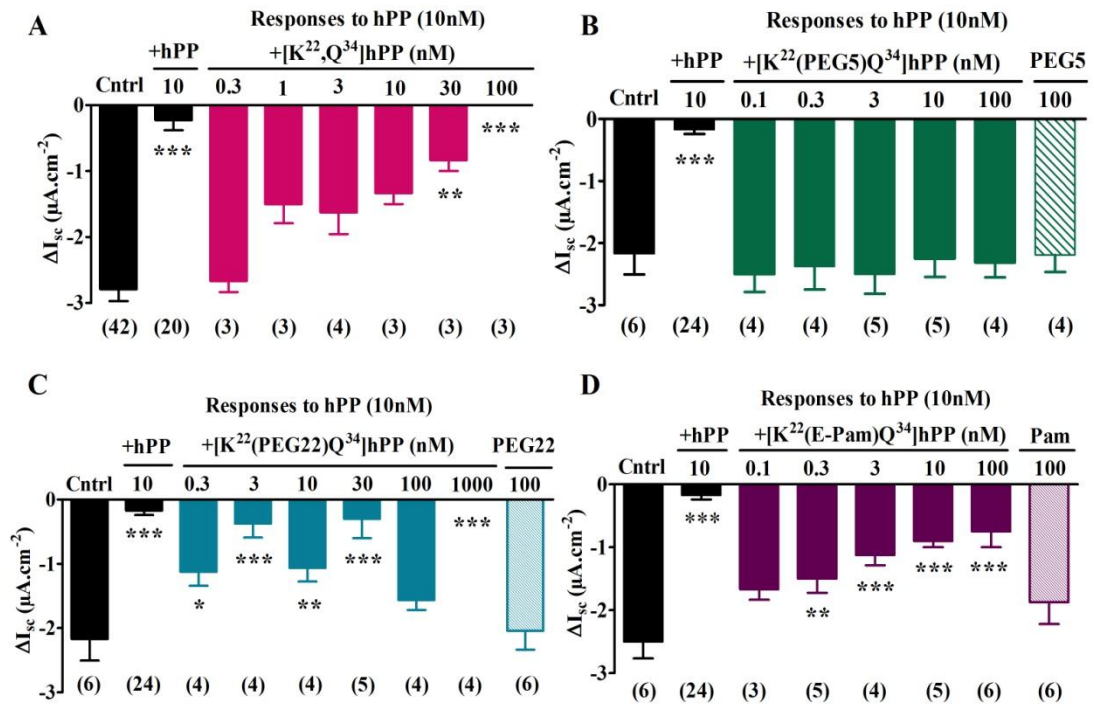
Peptide	EC <sub>50</sub> value (nM)	95% confidence
[Q <sup>34</sup> ]hPP	36.0	18.0-73.0
[K <sup>22</sup> , Q <sup>34</sup> ]hPP	8.4	2.4-29.0
[K <sup>22</sup> (PEG5)Q <sup>34</sup> ]hPP	0.9	0.3-2.1
[K <sup>22</sup> (PEG22)Q <sup>34</sup> ]hPP	>1 μM	
[K <sup>22</sup> (E-Pam)Q <sup>34</sup> ]hPP	9.6	4.5-20.0

**Table 3.4 Comparison of chemically modified [K<sup>22</sup>, Q<sup>34</sup>]hPP EC<sub>50</sub> values (with 95% confidence limits).** Data was calculated from non-cumulative concentration-response curves obtained from Col-24 monolayers shown in Figure 3.20.

### 3.4.3 Desensitisation of hPP-activated Y<sub>4</sub> responses with [K<sup>22</sup>, Q<sup>34</sup>]hPP modified analogues

As observed with hPP and [Q<sup>34</sup>]hPP, additions of the non-modified [K<sup>22</sup>, Q<sup>34</sup>]hPP in Col-24 monolayers resulted in a concentration-dependent reduction in subsequent hPP responses, which were significantly reduced following 30 nM and 100 nM [K<sup>22</sup>, Q<sup>34</sup>]hPP (Figure 3.21A). Chemically modified [K<sup>22</sup>, Q<sup>34</sup>]hPP variants exhibited similar profiles to those of the PEG-/lipid-modified [K<sup>30</sup>, Q<sup>34</sup>]hPP variants in terms of Y<sub>4</sub> receptor desensitisation. As seen with [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP, there was a significant lack of Y<sub>4</sub> desensitisation following [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP additions (0.1-100 nM) (Figure 3.21B), indicating that modification with PEG5 permits repeated Y<sub>4</sub> receptor activation without subsequent Y<sub>4</sub> receptor desensitisation.

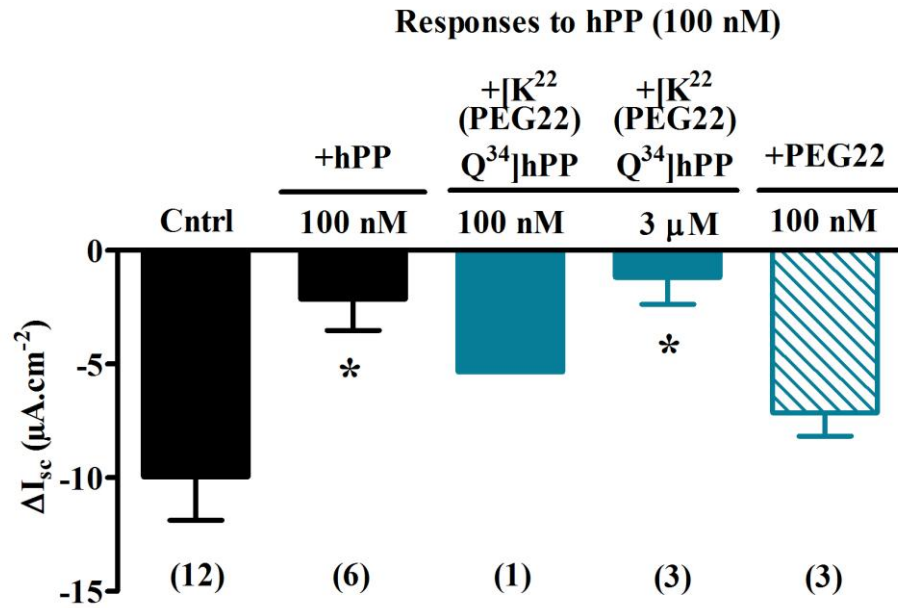
In contrast, [K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP resulted in significant Y<sub>4</sub> desensitisation at a concentration range of 0.3-100 nM (Figure 3.21C), an effect that was analogous to that seen with [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP (although this was not a concentration-dependent effect). Unexpectedly, subsequent hPP responses were significantly diminished after 0.3 nM-1 μM [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP (Figure 3.21D), despite complete inability of the peptide to reduce I<sub>sc</sub> (in contrast to [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP) in both Col-24 monolayers and human colon mucosa. As mentioned previously, the PEG5 and PEG22 polymers alone along with palmitic acid had no effect upon Y<sub>4</sub> receptor desensitisation.



**Figure 3.21 Desensitisation of hPP-activated responses following pre-treatment with chemically modified [K<sup>22</sup>, Q<sup>34</sup>]hPP variants in Col-24 monolayers.**

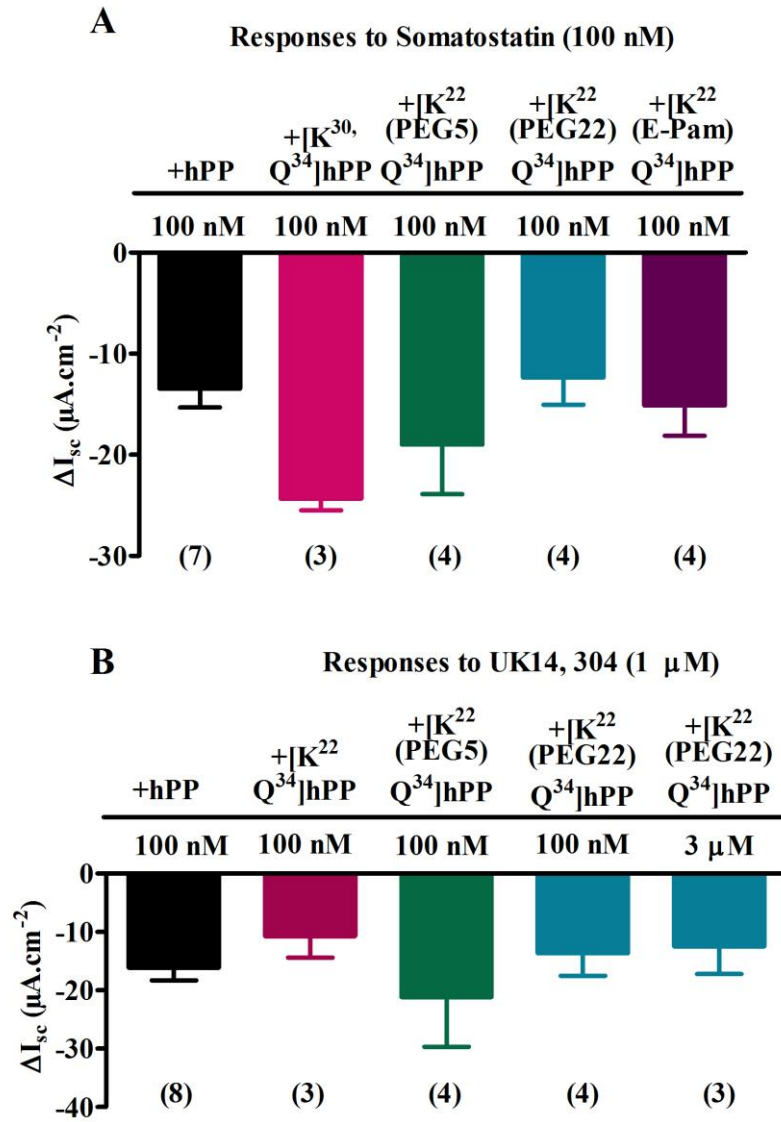
Desensitisation of hPP responses (10 nM throughout) added 45 min after increasing concentrations of either (A) [K<sup>22</sup>, Q<sup>34</sup>]hPP, (B) [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP, (C) [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP or (D) [K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP, showing differential desensitisation of subsequent hPP responses. Responses to hPP following different PEG or palmitic acid side chains are shown to the right of each histogram (hatched bars). Each bar represents the mean  $\pm$  1 S.E.M with *n* numbers shown in parenthesis. Statistical differences from 'Cntrl' hPP responses were determined using ANOVA and Dunnett's post-test, where \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ .

hPP desensitisation was also observed in human colonic specimens treated with [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP (100 nM and 3 μM, Figure 3.22), despite the PEG22 variant being inactive for up to 60 min after addition.



**Figure 3.22 Desensitisation of hPP responses following pre-treatment with  $[K^{22}(PEG22)Q^{34}]hPP$  in human colon mucosa.** Desensitisation of hPP responses (100 nM throughout) added 60 min after the addition of  $[K^{22}(PEG22)Q^{34}]hPP$  (100 nM and 3  $\mu M$ ) and the PEG22 polymer (100 nM, hatched bar). Each bar represents the mean  $\pm$  1 S.E.M with *n* numbers shown in parenthesis. Statistical differences from ‘Cntrl’ hPP responses were determined using ANOVA and Dunnett’s post-test, where \* *P* < 0.05.

As with the  $[K^{30}, Q^{34}]hPP$  variants, each modified  $[K^{22}, Q^{34}]hPP$  peptide demonstrated receptor specificity in their actions as subsequent  $G_{ai}$ -coupled somatostatin responses were not significantly affected in Col-24 monolayers (Figure 3.23A) and neither were  $G_{ai}$ -coupled  $\alpha$ -adrenoceptor responses, as determined by UK 14,304 responses in human colon mucosa (Figure 3.23B).



**Figure 3.23 Responses to somatostatin and UK 14,304 in Col-24 monolayers and human colon mucosa, respectively, following pre-treatment with PEG/lipid-modified  $[K^{22}, Q^{34}]hPP$  variants.** Selectivity of  $G_{ai}$ - coupled signalling demonstrated by insignificant changes in the response to somatostatin (100 nM) after pre-treatment with (A) hPP,  $[K^{22}, Q^{34}]hPP$ ,  $[K^{22}(PEG5)Q^{34}]hPP$ ,  $[K^{22}(PEG22)Q^{34}]hPP$  and  $[K^{22}(E-Pam)Q^{34}]hPP$ . (B) Responses to UK 14,304 (1  $\mu$ M) after pre-treatment of the same peptides in human colon mucosa. Each bar represents the mean  $\pm$  1 S.E.M with  $n$  numbers shown in parenthesis. There were no statistical differences from control hPP responses as determined by ANOVA with Dunnett's post-test.

#### 3.4.4 Summary of results with $[K^{30}, Q^{34}]hPP$ and $[K^{22}, Q^{34}]hPP$ analogues

As shown previously, responses to Y agonists in Col-24 epithelial monolayers were anti-secretory via activation of basolateral  $Y_4$  receptors (Cox *et al.*, 2001b) and were dependent on VIP stimulated ion secretion. Utilising Col-24 monolayers in this study, anti-secretory responses to hPP were transient, with peak responses being reached at approximately 5 min. The dual  $Y_2/Y_4$  receptor agonist,  $[Q^{34}]hPP$ , displayed a

comparable rapid time course profile to that of hPP, and selective blockade of Y<sub>2</sub> receptors using a competitive Y<sub>2</sub> antagonist revealed the dual capacity of this analogue in human colonic mucosa. Modification at position 22 with lysine was well tolerated, demonstrated by increased Y<sub>4</sub> potency.

With regard to the [K<sup>30</sup>, Q<sup>34</sup>]hPP series of peptides, the addition of a PEG polymer resulted in responses that were delayed in onset, and the extent of delay correlated with the size of the PEG, where PEG22 > PEG5 > PEG2. PEG5 conjugation seemed to be well tolerated with significantly increased efficacy compared with the PEG2 and the non-modified [Q<sup>34</sup>]hPP, as observed in Figure 3.14. Palmitoylation of [Q<sup>34</sup>]hPP was also well tolerated in terms of efficacy and potency and displayed equi-effective responses compared to the PEG5-conjugated peptide in Col-24 epithelial layers. Importantly, despite having nM selectivity for the Y<sub>1</sub> receptor (indicated by IP<sub>3</sub> signalling), the palmitoylated dual [K<sup>30</sup>, Q<sup>34</sup>]hPP derivative demonstrated significant Y<sub>2</sub> and Y<sub>4</sub> functional output in human colon mucosa with no significant Y<sub>1</sub> activity, revealing high Y<sub>2</sub> and Y<sub>4</sub> receptor functional output for the novel peptide. The addition of a lysine linker at position 22 also enabled successful PEG and lipid conjugation, as determined by their prolonged anti-secretory responses and well preserved potency, with the exception of PEG22 conjugation, which was unfavourable to both potency and efficacy in Col-24 monolayers and human colon mucosa. Epithelial monolayers pre-treated with [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP exhibited an unexpected resistance to hPP-activated desensitisation which was also consistent with [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP. On the contrary, palmitoylation of both peptide variants seemed to facilitate desensitisation, revealed by the significant loss of further Y<sub>4</sub> signalling in Col-24 epithelia, even after low nM concentrations of palmitoylated agonist. Interestingly, both PEG22-ylated variants resulted in partial inhibition of subsequent hPP responses, despite a complete lack of biological activity with [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP.

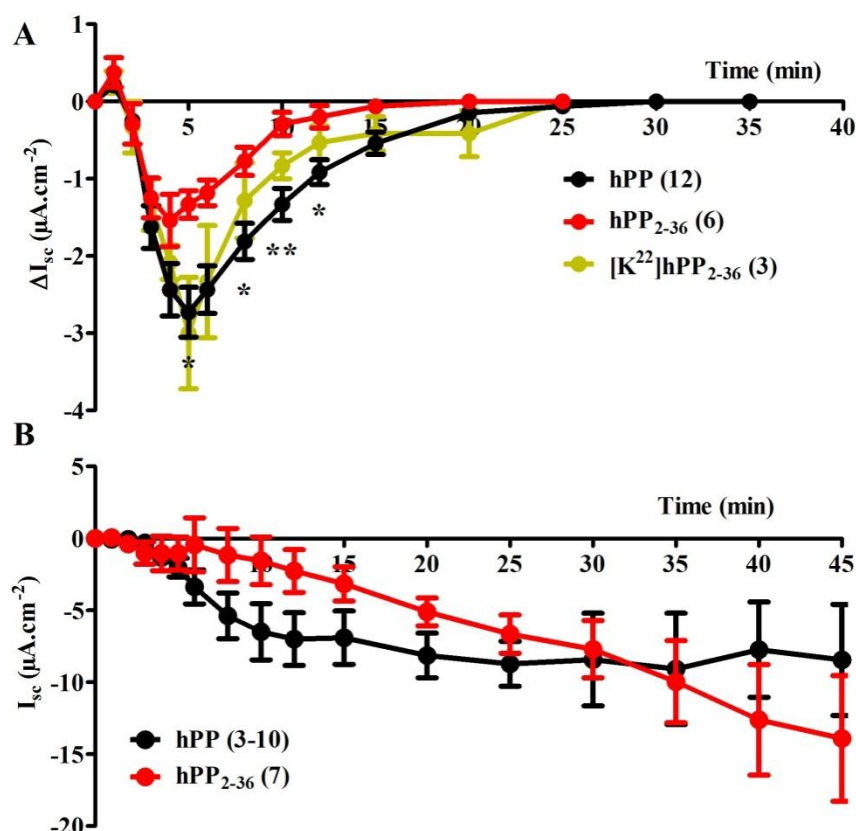
### 3.5 Functional assessment of TM30339

TM30339 (hPP<sub>2-36</sub>), is a Y<sub>4</sub> selective agonist with improved stability compared to hPP. The native hormone is normally a substrate for DPP-IV degradation due to the presence of Pro at position 2. Thus, by removing the first residue, a peptide is generated which is no longer a substrate for DPP-IV (European patent application, reference no. EP2060266B1, 2005). Importantly, the Y<sub>4</sub> receptor is not dependent on the residue

located at position 1, unlike the Y<sub>1</sub> receptor, thus hPP<sub>2-36</sub> has increased selectivity against the Y<sub>1</sub> receptor.

### **3.5.1 Time course profiles of epithelial and mucosal I<sub>sc</sub> responses to hPP<sub>2-36</sub> and [K<sup>22</sup>]hPP<sub>2-36</sub>**

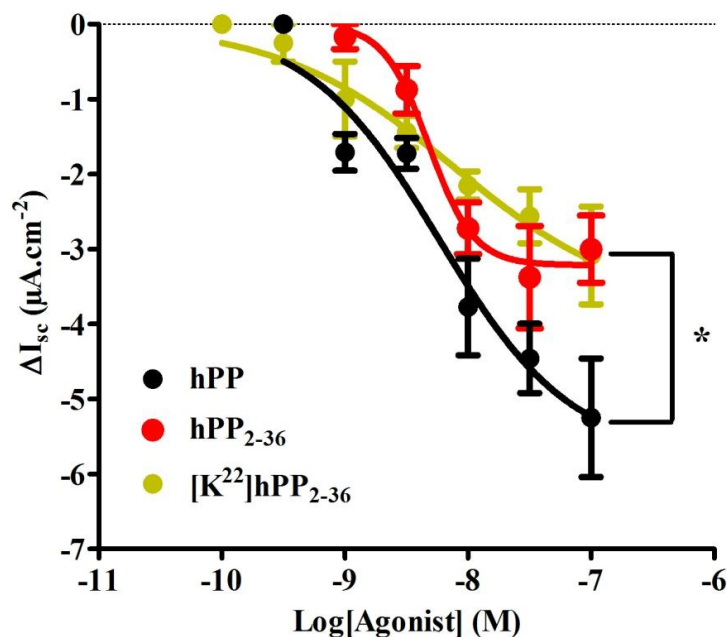
In Col-24 monolayers, responses to single additions of hPP<sub>2-36</sub> (100 nM) were transient, reducing I<sub>sc</sub> with a peak response at 4 min ( $-1.5 \pm 0.3 \mu\text{A} \cdot \text{cm}^{-2}$ ;  $n=6$ ). This profile was comparable to 10 nM hPP, peaking at 5 min ( $-2.7 \pm 0.3 \mu\text{A} \cdot \text{cm}^{-2}$ ;  $n=12$ ). Modification at position 22 with lysine ([K<sup>22</sup>]hPP<sub>2-36</sub>) was well tolerated and resulted in maximal response of  $-3.0 \pm 0.7 \mu\text{A} \cdot \text{cm}^{-2}$ ;  $n=3$ ), reaching a peak at 5 min. No significant difference was observed in the rate of onset between the peptides, however hPP generated a larger response size compared to hPP<sub>2-36</sub> (Figure 3.24A). In human colon mucosa, a 100 nM concentration of hPP and hPP<sub>2-36</sub> revealed no significant differences between these two in terms of maximum peak response, although a slightly faster rate of onset was observed for hPP (Figure 3.24).



**Figure 3.24 Comparison of the anti-secretory responses to hPP<sub>2-36</sub> and [K<sup>22</sup>]hPP<sub>2-36</sub>.** Time course profiles of pooled anti-secretory responses in (A) Col-24 monolayers and (B) human colon mucosa. hPP<sub>2-36</sub> and [K<sup>22</sup>]hPP<sub>2-36</sub> (100 nM in both preparations) and hPP (10 nM in Col-24 monolayers and 100 nM in human mucosa) were added basolaterally at  $t = 0$  min. Data are presented as means  $\pm$  1 SEM, while  $n$  numbers are indicated in parenthesis. Statistical significances between hPP and hPP<sub>2-36</sub> were determined using a one way ANOVA with Bonferroni's post-test, where \*  $P < 0.05$  and \*\*  $P < 0.01$ . One way ANOVA with Dunnett's post-test determined no significant difference between hPP and hPP<sub>2-36</sub> in human colon mucosa.

### 3.5.2 Concentration response relationships for hPP<sub>2-36</sub> and [K<sup>22</sup>]hPP<sub>2-36</sub>

Responses to hPP<sub>2-36</sub> and [K<sup>22</sup>]hPP<sub>2-36</sub> were concentration-dependant in Col-24 monolayers and the resulting concentration-response curves produced equipotent EC<sub>50</sub> values of 4.8 nM (95% confidence, 2.5 - 9.1) and 7.6 nM (95% confidence, 4.4-13.0), respectively (Figure 3.25).

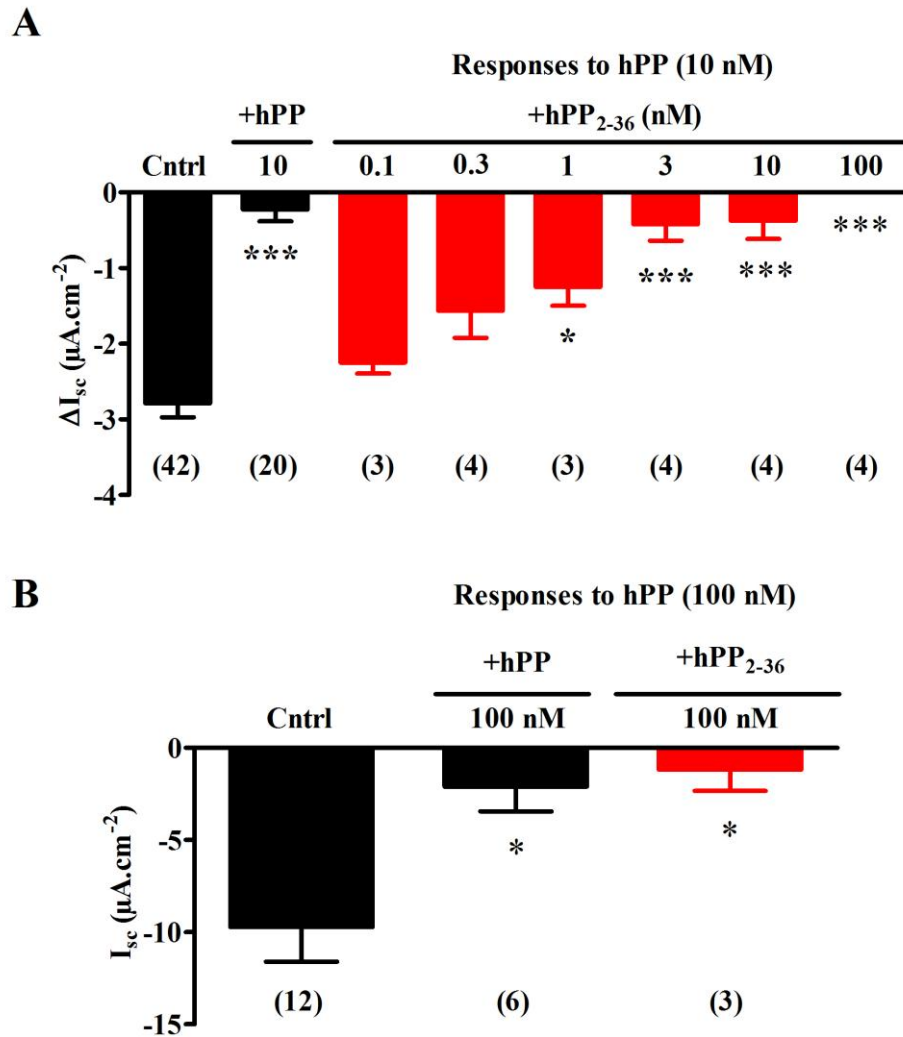


**Figure 3.25 Concentration-response profiles for hPP<sub>2-36</sub> and [K<sup>22</sup>]hPP<sub>2-36</sub> compared with hPP.** Non-cumulative pooled responses to hPP ( $n=3-21$ ), hPP<sub>2-36</sub> ( $n=3-9$ ) and [K<sup>22</sup>]hPP<sub>2-36</sub> ( $n=3-5$ ) in Col-24 monolayers, generating EC<sub>50</sub> values quoted in the text. Each point represents the mean  $\pm$  1 S.E.M. of pooled data. One way ANOVA with Bonferroni's post-test determined a significant difference between hPP and hPP<sub>2-36</sub>,  $P < 0.05$

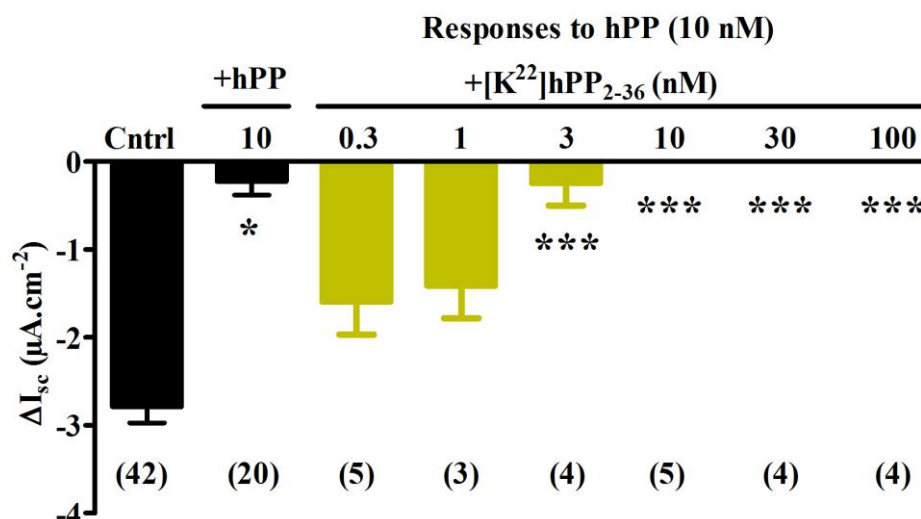
### 3.5.3 Desensitisation of hPP responses following pre-treatment with hPP<sub>2-36</sub> and [K<sup>22</sup>]hPP<sub>2-36</sub>

Concentration-dependent reductions to subsequent hPP (10 nM) responses after pre-treatment with increasing concentrations of hPP<sub>2-36</sub> (0.1 nM – 100 nM) were observed, with 1 nM hPP<sub>2-36</sub> being the threshold effective concentration to cause significant Y<sub>4</sub> receptor desensitisation ( $P < 0.05$ ) (Figure 3.26A). This effect was analogous to that seen with the native hormone hPP. Likewise, in human mucosa, 100 nM hPP<sub>2-36</sub> also caused significant reductions in subsequent hPP responses (100 nM,  $P < 0.05$ ) (Figure 3.26B). [K<sup>22</sup>]hPP<sub>2-36</sub> also induced concentration-dependent reductions of hPP responses in Col-24 monolayers, with significant desensitisation observed with 3 nM [K<sup>22</sup>]hPP<sub>2-36</sub> ( $P < 0.001$ ) (Figure 3.27) and 10 nM [K<sup>22</sup>]hPP<sub>2-36</sub> or above resulted in a complete loss in response to hPP.





**Figure 3.26 Desensitisation of hPP activated  $Y_4$  responses by hPP<sub>2-36</sub> in Col-24 monolayers and colon mucosa.** (A) Desensitisation of hPP responses in Col-24 monolayers (10 nM throughout) added 25 min after increasing concentrations of hPP<sub>2-36</sub>. In (B) desensitisation of hPP responses (100 nM) in human colon mucosa, added 60 min after hPP<sub>2-36</sub> (100 nM). Each bar represents the mean  $\pm$  1 S.E.M with *n* numbers shown in parenthesis. Statistical differences from control hPP responses were determined using ANOVA and Dunnett's post-test, where \*  $P < 0.05$ , \*\*\*  $P < 0.001$ .



**Figure 3.27 Desensitisation of the hPP activated  $Y_4$  responses by  $[K^{22}]hPP_{2-36}$ .** Responses to hPP (10 nM) added 25 min after pre-treatment with increasing concentration of  $[K^{22}]hPP_{2-36}$  (0.3 nM-100 nM) in Col-24 monolayers. Each bar represents the mean  $\pm$  1 S.E.M and *n* values are shown in parentheses. \*\*\* *P* < 0.001 and \* *P* < 0.05 compared with control hPP ('Cntrl', 10 nM) responses, measured using a one-way ANOVA and Dunnett's post-test.

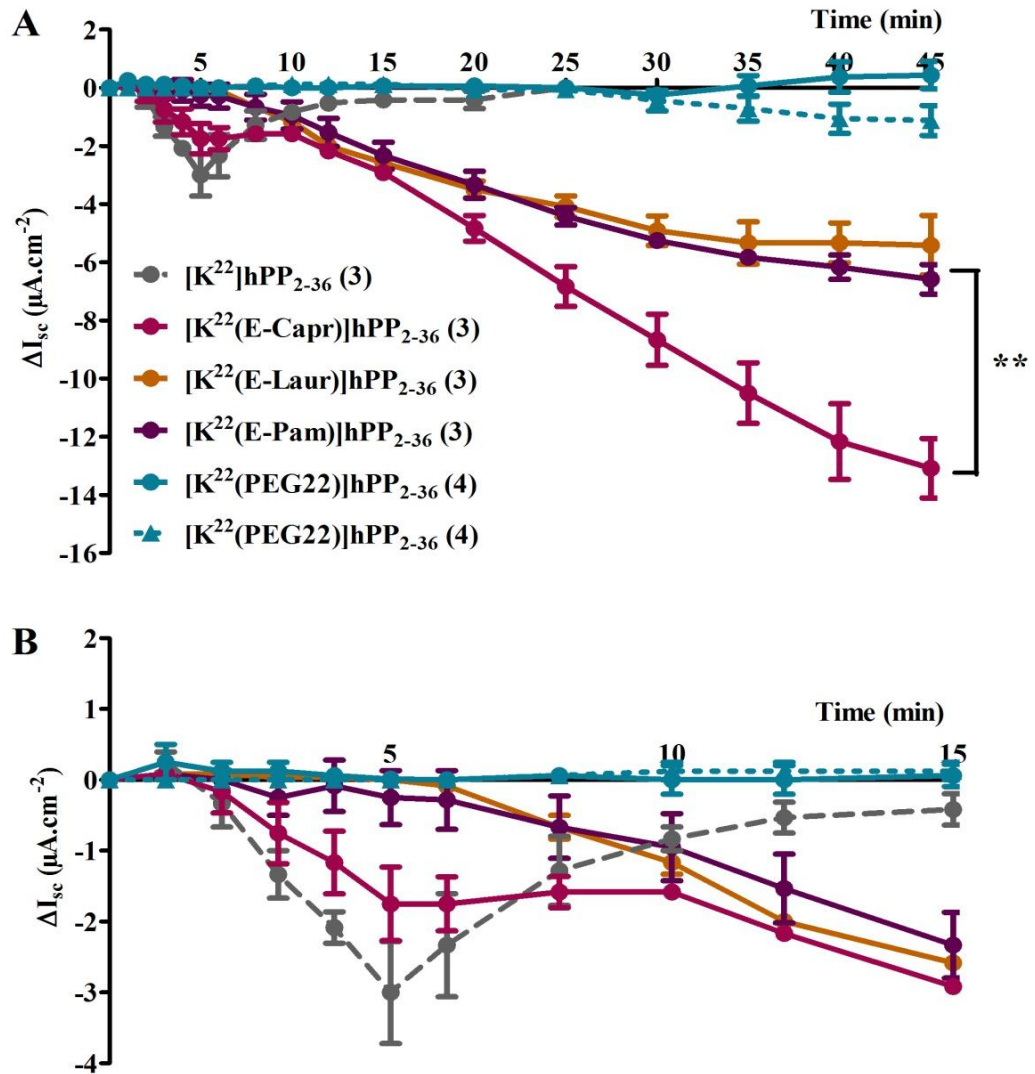
### 3.6 Functional assessment of fatty acid- and PEG conjugated $[K^{22}]hPP_{2-36}$ analogues

Since modification of  $[Q^{34}]hPP$  with palmitic acid demonstrated an improved *in vitro* pharmacological profile, the  $[K^{22}]hPP_{2-36}$  variants were chemically conjugated with a short chain (C8), medium chain (C12) and long chain (C16) fatty acid moiety and investigated for enhanced anti-secretory capabilities in both colon preparations. Given that PEG22 modification of the  $[Q^{34}]hPP$  series led to a loss in biological activity, reductions in  $I_{sc}$  were measured with  $[K^{22}(PEG22)]hPP_{2-36}$  to discern whether modification at a different site on the hPP sequence would improve the anti-secretory profile.

#### 3.6.1 Time course profiles of epithelial and mucosal $I_{sc}$ responses to lipidated and PEGylated $[K^{22}]hPP_{2-36}$ analogues

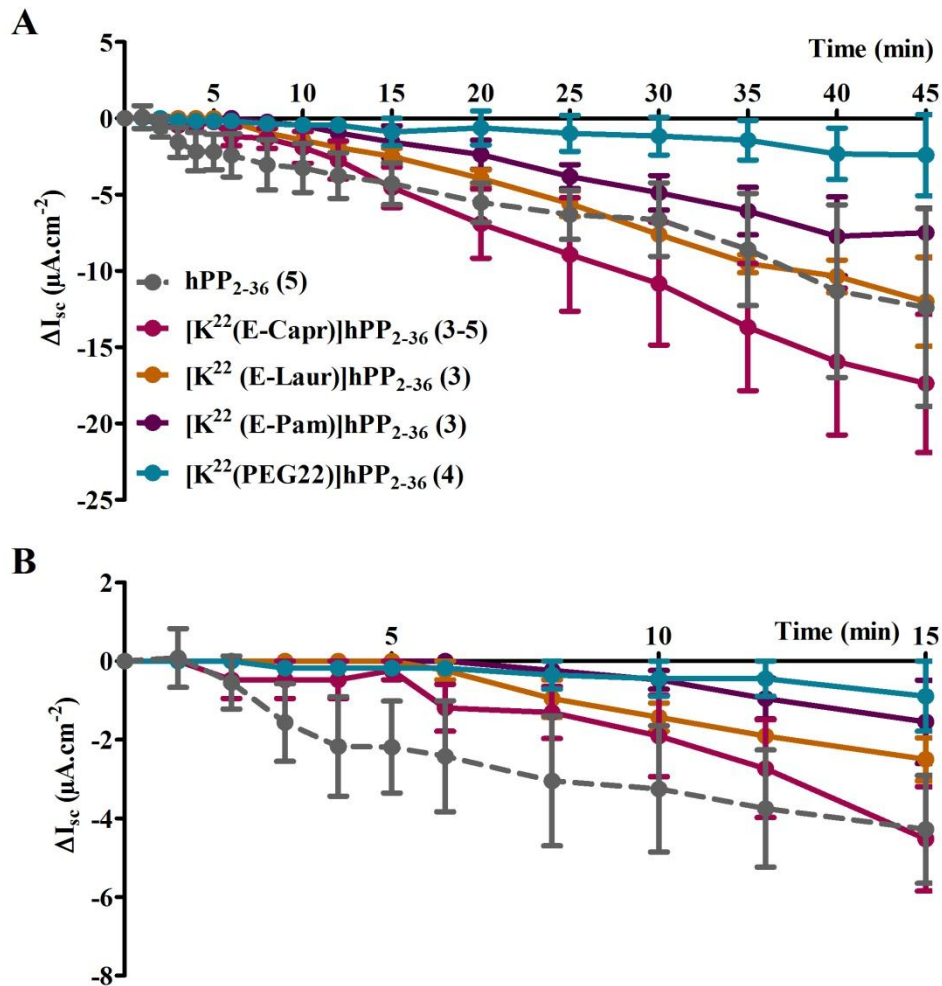
All three lipidated peptides were found to inhibit VIP-stimulated  $Cl^-$  secretion in Col-24 monolayers, the addition of  $[K^{22}(E-Laur)]hPP_{2-36}$  and  $[K^{22}(E-Pam)]hPP_{2-36}$  (100 nM) resulted in monophasic prolonged anti-secretory responses which were equi-effective at 45 min (Figure 3.28A). Both  $[K^{22}(E-Laur)]hPP_{2-36}$  and  $[K^{22}(E-Pam)]hPP_{2-36}$  displayed a slower onset of action compared with the non lipidated  $[K^{22}]hPP_{2-36}$  (Figure 3.28B). Interestingly,  $[K^{22}(E-Capr)]hPP_{2-36}$  (100 nM) displayed a biphasic anti-secretory

response, with an initial transient phase that appeared to peak at 5 min ( $-1.8 \pm 0.5$   $\mu\text{A}\cdot\text{cm}^{-2}$ ;  $n=3$ ), resembling that of the unmodified  $[\text{K}^{22}]\text{hPP}_{2-36}$ , followed by a sustained anti-secretory response. This second phase appeared at  $\sim 10$  min and peaked within 45 min, additionally displaying significantly enhanced response size relative to  $[\text{K}^{22}(\text{E-Laur})]\text{hPP}_{2-36}$  and  $[\text{K}^{22}(\text{E-Pam})]\text{hPP}_{2-36}$ . Modification of  $[\text{K}^{22}]\text{hPP}_{2-36}$  with a PEG22 polymer resulted in a complete loss of biological activity. No reduction in  $I_{\text{sc}}$  was observed of this large peptide (100 nM, or 3  $\mu\text{M}$ ) during the observation period (Figure 3.28).



**Figure 3.28 Time course profiles of chemically modified  $[\text{K}^{22}]\text{hPP}_{2-36}$  variants in Col-24 monolayers.** In (A) reductions in  $I_{\text{sc}}$  in response to (100nM) of  $[\text{K}^{22}(\text{E-Capr})]\text{hPP}_{2-36}$ ,  $[\text{K}^{22}(\text{E-Laur})]\text{hPP}_{2-36}$ ,  $[\text{K}^{22}(\text{E-Pam})]\text{hPP}_{2-36}$  and  $[\text{K}^{22}(\text{PEG22})]\text{hPP}_{2-36}$  (100 nM and 3  $\mu\text{M}$  – dashed line) are shown for a period of 45 min, demonstrating loss of activity with a PEG22 modification. (B) A magnification of early time points between 0 and 15 min for each modified peptide. Data are presented as means  $\pm$  1 SEM, while  $n$  numbers are indicated in parenthesis. Multiple comparisons were made using one-way ANOVA with Bonferroni's post-test,  $** P < 0.01$ , compared with each analogue.

In colonic mucosal specimens, lipidated analogues displayed similar patterns in terms of efficacy, with  $[K^{22}(\text{E-Laur})]hPP_{2-36}$  and  $[K^{22}(\text{E-Pam})]hPP_{2-36}$  demonstrating comparable rates of onset and time to peak, both of which were monophasic in nature (Figure 3.29).  $[K^{22}(\text{E-Capr})]hPP_{2-36}$  also displayed the biphasic nature observed in Col-24 monolayers with an initial transient peak and a subsequent sustained anti-secretory response. Again, the anti-secretory ability of  $[K^{22}(\text{E-Capr})]hPP_{2-36}$  was greater than that seen with longer fatty acids (Figure 3.29), however, in human colon mucosa a one way ANOVA with Bonferroni's post-test did not show any significant differences between the peak responses of the peptides. As observed in Col-24 monolayers, little anti-secretory activity was observed with  $[K^{22}(\text{PEG22})]hPP_{2-36}$  in isolated mucosa (Figure 3.29).

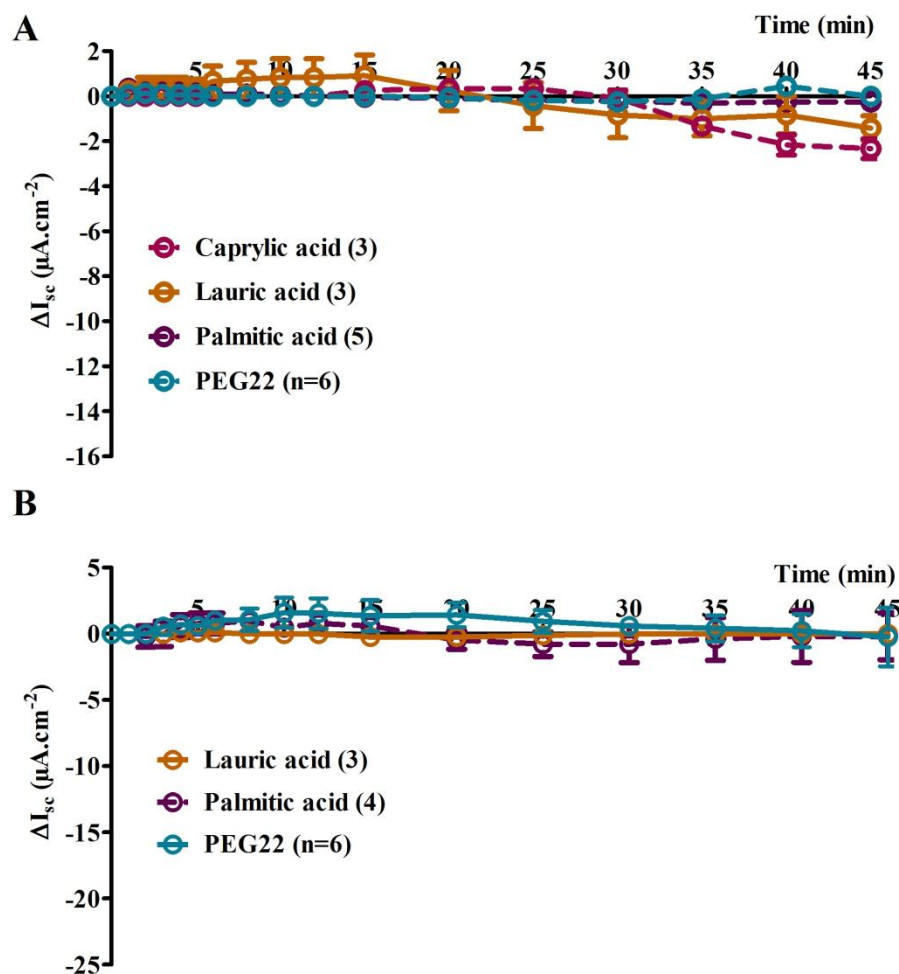


**Figure 3.29 Anti-secretory responses to chemically modified  $[K^{22}]hPP_{2-36}$  variants in human colon mucosa.** (A) Reductions in  $I_{sc}$  in response to  $[K^{22}(\text{E-Capr})]hPP_{2-36}$ ,  $[K^{22}(\text{E-Laur})]hPP_{2-36}$ ,  $[K^{22}(\text{E-Pam})]hPP_{2-36}$  and  $[K^{22}(\text{PEG22})]hPP_{2-36}$  (100 nM) are shown for a period of 45 min, demonstrating reduced efficacy following a PEG22 modification. (B) A magnification of early time points between 0 and 15 min for each modified peptide. Data are presented as means  $\pm$  1 SEM, while  $n$  numbers are indicated

in parenthesis. Multiple comparisons were made using one-way ANOVA with Bonferroni's post-test.

### 3.6.2 Fatty acid responses in epithelial and mucosal preparations

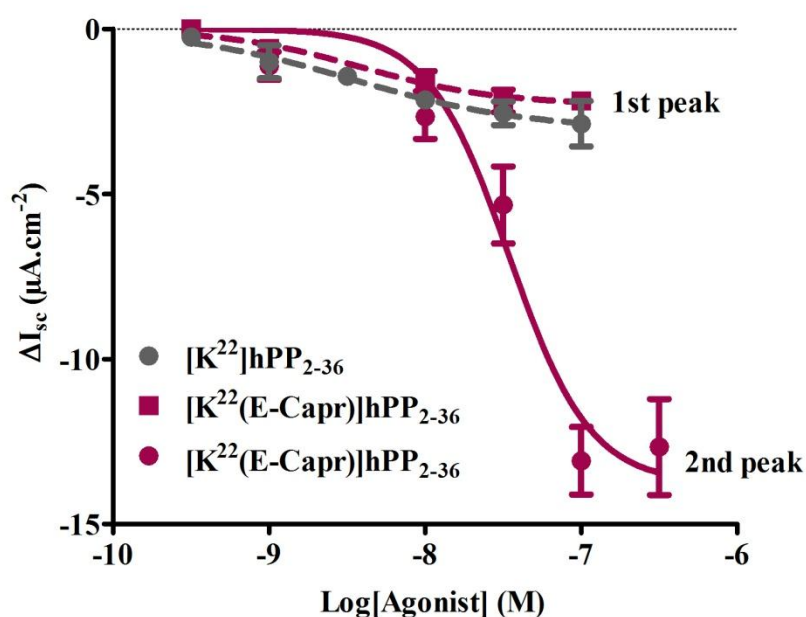
It is well documented that the release of PYY and GLP-1 from enteroendocrine L cells can be activated by a range of luminal nutrients such as fatty acids (Anini *et al.*, 1999; Hirasawa *et al.*, 2005). Limited published data has reported that both GPR40 (Itoh *et al.*, 2003) and GPR120 (Hirasawa *et al.*, 2005) specifically detect long-chain fatty acids, and for this reason, the response to the fatty acid side chains were assessed in both preparations and compared with the PEG22 side chain. This was to determine whether reductions in  $I_{sc}$  were an indirect effect of the fatty acids and to rule out the possibility of PYY-mediated  $Y_1$  anti-secretory responses in human colon mucosa. In both colonic preparations, all fatty acid side chains had no effects upon  $I_{sc}$  levels (Figure 3.30).



**Figure 3.30 Anti-secretory responses to fatty acid side chains and PEG22.** Lack of change in  $I_{sc}$  to caprylic acid, lauric acid, palmitic acid and PEG22 (100 nM) following VIP (30 nM) stimulation in (A) Col-24 monolayers and (B) human colon mucosa. Data are presented as means  $\pm$  1 SEM, while  $n$  numbers are indicated in parenthesis.

### 3.6.3 Concentration response relationships for $[K^{22}(E\text{-Capr})]hPP_{2-36}$ , $[K^{22}(E\text{-Laur})]hPP_{2-36}$ , $[K^{22}(E\text{-Pam})]hPP_{2-36}$ and $[K^{22}(PEG22)]hPP_{2-36}$ in Col-24 monolayers

The ability of  $[K^{22}(E\text{-Capr})]hPP_{2-36}$  to inhibit  $Cl^-$  secretion was biphasic in nature, as demonstrated by a pooled time course profile in Col-24 monolayers. The concentration response relationship indicated anti-secretory activity being maximal between 100 and 300 nM (Figure 3.31). Segregating the two components of the  $[K^{22}(E\text{-Capr})]hPP_{2-36}$  response demonstrated an initial transient phase, generated an  $EC_{50}$  value of 3.9 nM (1.2 - 13.0 nM), which did not significantly differ from  $[K^{22}]hPP_{2-36}$  (3.4 nM, 0.6 - 18.0 nM). The second prolonged anti-secretory phase had a 10 fold lower  $EC_{50}$  value of 35.0 nM (22.0-56.0 nM) however a much greater efficacy; the size of the peak response to  $[K^{22}(E\text{-Capr})]hPP_{2-36}$  (100 nM) during the 2<sup>nd</sup> phase was 83.2% larger compared with the maximal response size during the 1<sup>st</sup> peak to the same peptide.

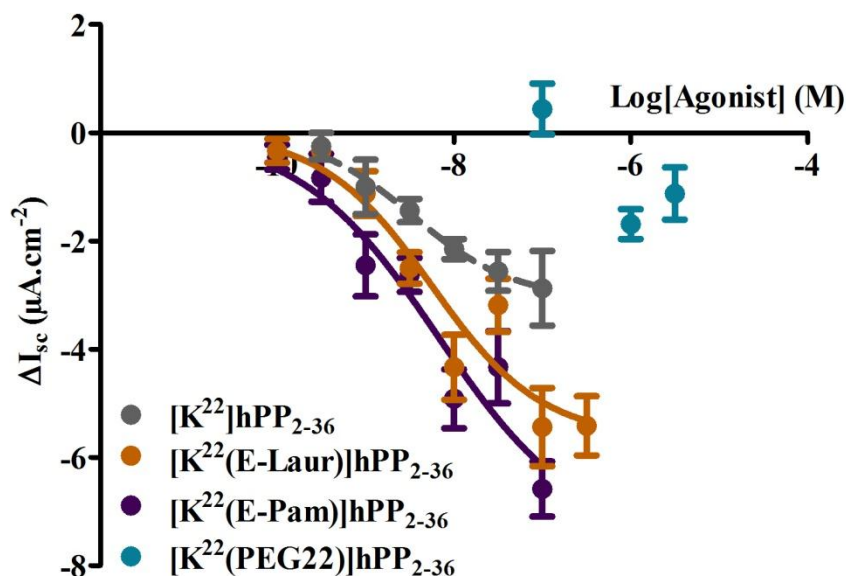


**Figure 3.31 Non-cumulative concentration-response profiles for  $[K^{22}]hPP_{2-36}$  and  $[K^{22}(E\text{-Capr})]hPP_{2-36}$ .** Responses to  $[K^{22}]hPP_{2-36}$  (measured at ~5 min) are shown with a grey dashed line. Anti-secretory responses to  $[K^{22}(E\text{-Capr})]hPP_{2-36}$  are shown, representing the size of the response in the first transient phase ( $n=4-7$ , measured at ~5 min, dashed pink line) compared with the subsequent prolonged phase ( $n=3-4$ , measured at ~45 min, unbroken pink line). All curves generated  $EC_{50}$  values and 95% confidence limits quoted in the text. Each point represents the mean  $\pm$  1 S.E.M of pooled data.

In addition to exhibiting protracted responses compared to  $[K^{22}]hPP_{2-36}$ , increasing the length of the fatty acid chain was not detrimental to agonist potency.  $[K^{22}(E\text{-Laur})]hPP_{2-36}$  and  $[K^{22}(E\text{-Pam})]hPP_{2-36}$  both retained the high potency observed with their non-lipidated predecessor (Figure 3.32), indicated by equipotent  $EC_{50}$  values (5.6 nM (1.4-



22.0 nM), compared with 6.6nM (0.5-79.0 nM) for  $[K^{22}(E-Pam)]hPP_{2-36}$ , respectively, both of which were increased compared with  $[K^{22}(E-Capr)]hPP_{2-36}$ . Modification of  $[K^{22}]hPP_{2-36}$  with PEG22 resulted in a significant loss of biological activity at concentrations ranging from 1 nM to 3  $\mu$ M.

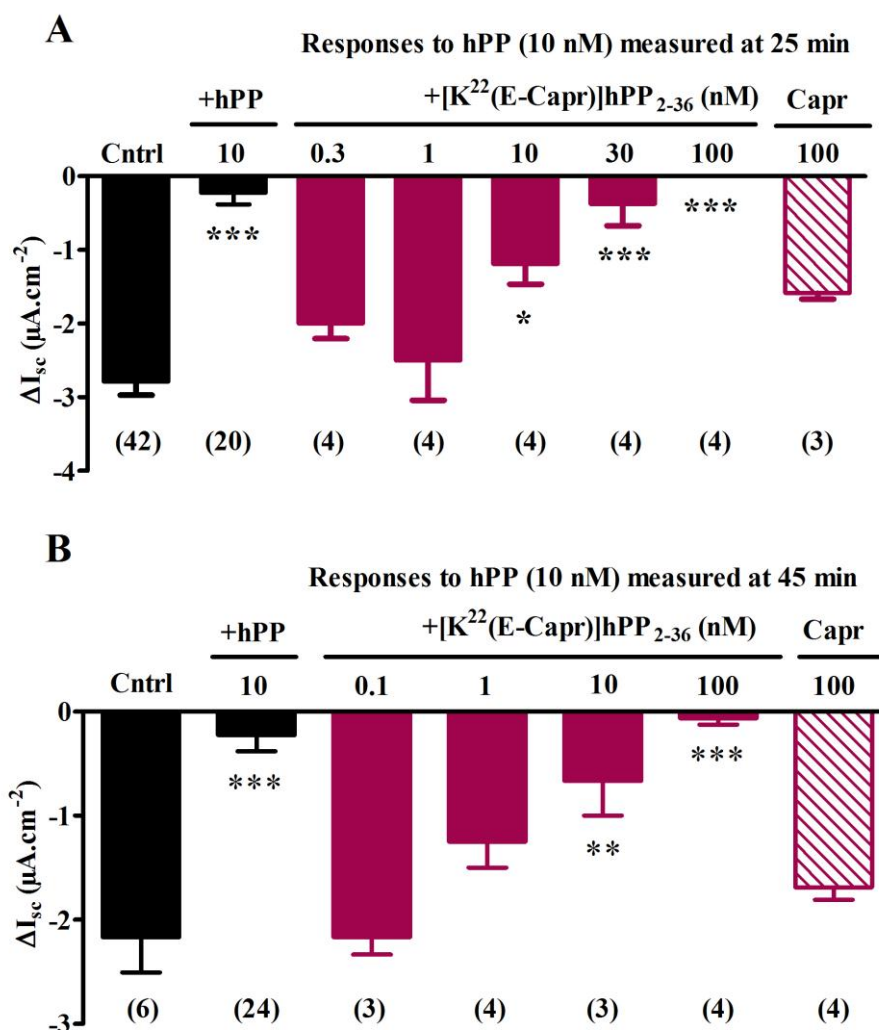


**Figure 3.32 Non-cumulative concentration-response profiles for  $[K^{22}(E-Laur)]hPP_{2-36}$ ,  $[K^{22}(E-Pam)]hPP_{2-36}$  and  $[K^{22}(PEG22)]hPP_{2-36}$  in Col-24 monolayers.** Anti-secretory responses to  $[K^{22}(E-Laur)]hPP_{2-36}$  ( $n=3-4$ ) and  $[K^{22}(E-Pam)]hPP_{2-36}$  ( $n=3-5$ ) are compared with the non lipidated  $[K^{22}]hPP_{2-36}$  peptide ( $n=3-5$ ). The response to 100 nM, 1  $\mu$ M and 3  $\mu$ M  $[K^{22}(PEG22)]hPP_{2-36}$  demonstrates lack of potency and efficacy compared with the lipidated variants. The peak response was measured at approximately 45 min compared with  $[K^{22}]hPP_{2-36}$  at 5 min (grey dashed line), generating  $EC_{50}$  values and 95% confidence limits quoted in the text. Each point represents the mean  $\pm$  S.E.M of pooled data.

#### 3.6.4 Desensitisation of hPP responses following pre-treatment with $[K^{22}(E-Capr)]hPP_{2-36}$ , $[K^{22}(E-Laur)]hPP_{2-36}$ and $[K^{22}(E-Pam)]hPP_{2-36}$

Desensitisation studies with these lipidated variants (at different concentrations) in Col-24 monolayers revealed a significant loss of further  $Y_4$  signalling indicated by reductions in subsequent hPP responses. Since  $[K^{22}(E-Capr)]hPP_{2-36}$  displayed a biphasic anti-secretory response, the size of the response to hPP (10 nM) was measured after the first phase (25 min) and second phase (45 min) following pre-treatment with increasing concentrations of  $[K^{22}(E-Capr)]hPP_{2-36}$  (0.1- 100 nM) to investigate whether the receptor remained sensitive despite continuing  $Y_4$  signalling. Reductions in subsequent hPP responses after pre-treatment with  $[K^{22}(E-Capr)]hPP_{2-36}$  were concentration-dependent (Figure 3.33) and responses remained desensitised 45 min after the addition of the lipidated peptide (10 nM,  $P < 0.01$ ), signifying a loss in further  $Y_4$  signalling despite an on-going downstream anti-secretory response. Importantly, hPP

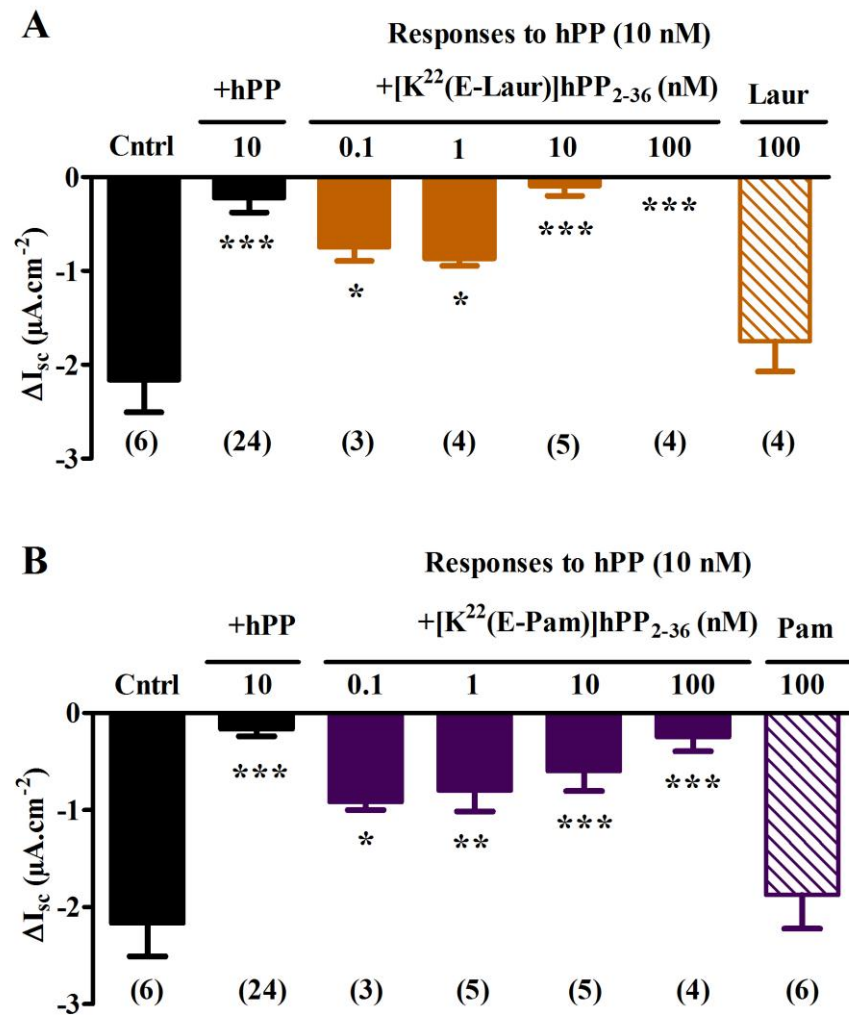
responses after addition of caprylic acid (100 nM) did not exhibit significant desensitisation compared with control hPP responses (see hatched bars to right of each histogram in Figure 3.33).



**Figure 3.33 Desensitisation of hPP activated responses after pre-treatment with a short chain fatty acid conjugated  $[K^{22}]hPP_{2-36}$  variants in Col-24 epithelial monolayers.** Desensitisation of hPP responses (10 nM throughout) added 25 min (A) or 45 min (B) after increasing concentrations of  $[K^{22}(E-Capr)]hPP_{2-36}$ , compared with  $Y_4$  desensitisation following hPP (10 nM). The response to hPP after pre-treatment with caprylic acid (Capr, 100 nM) is shown to the right of both histograms. Each bar represents the mean  $\pm$  1 S.E.M of pooled data with *n* numbers shown in parenthesis. Statistical differences from ‘Cntrl’ hPP responses were determined using ANOVA and Dunnett’s post-test, where \*  $P < 0.05$ , \*\*  $P < 0.01$  \*\*\*  $P < 0.001$ .

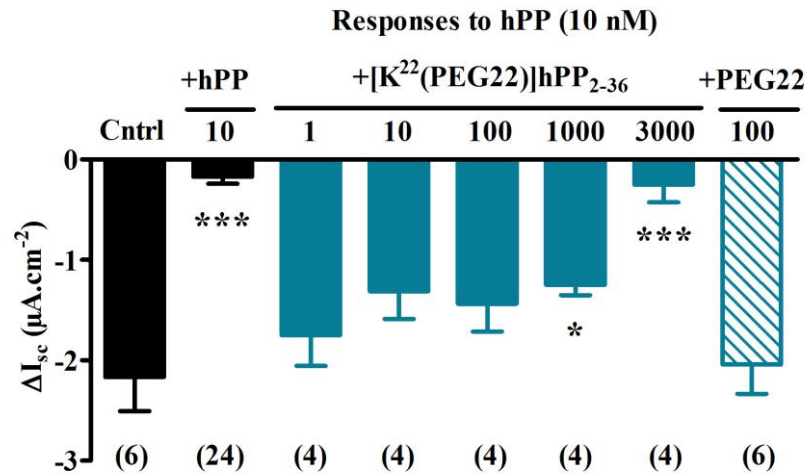
Pre-treatment of Col-24 monolayers with  $[K^{22}(E-Laur)]hPP_{2-36}$  or  $[K^{22}(E-Pam)]hPP_{2-36}$  (0.1-100 nM) led to significantly reduced hPP responses at concentrations as low as 0.1nM, suggesting high potency activation of the  $Y_4$  receptor and subsequent desensitisation with the longer fatty acid-conjugated peptides. Lauric acid and palmitic acid did not apparently cause significant  $Y_4$  receptor desensitisation (Figure 3.34).





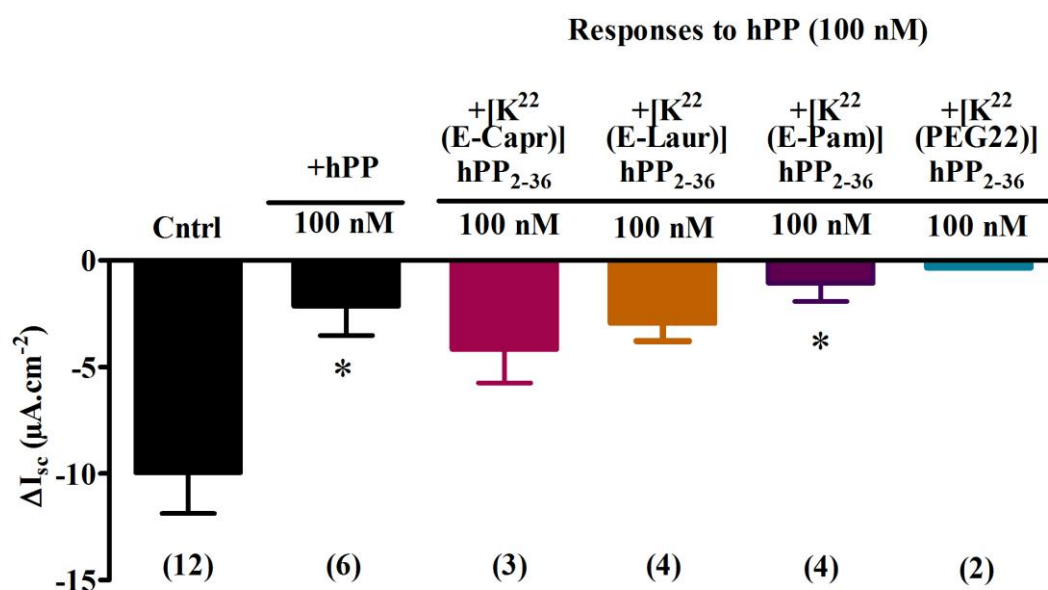
**Figure 3.34 Desensitisation of hPP activated responses following pre-treatment with lauric acid and palmitic acid conjugated [K<sup>22</sup>]hPP<sub>2-36</sub> variants in Col-24 epithelial monolayers.** Desensitisation of hPP responses (10 nM throughout) added 45 min after increasing concentrations of either [K<sup>22</sup>(E-Laur)]hPP<sub>2-36</sub> and [K<sup>22</sup>(E-Pam)]hPP<sub>2-36</sub>, compared with Y<sub>4</sub> desensitisation following hPP (100 nM). Responses to hPP after pre-treatment with lauric acid (Laur) and palmitic acid (Pam) are shown to the right of each histogram. Each bar represents the mean  $\pm$  1 S.E.M of pooled data with *n* numbers shown in parenthesis. Statistical differences from 'Cntrl' hPP responses were determined using ANOVA and Dunnett's post-test, where \* *P* < 0.05, \*\* *P* < 0.01 \*\*\* *P* < 0.001.

Unlike the lipidated variants, the PEGylated low potency agonist, [K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub> (1-100 nM), did not reduce subsequent hPP responses in Col-24 monolayers (Figure 3.35), as expected. A concentration of 1 μM however, significantly desensitised subsequent hPP responses and at 3 μM [K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub> even more so, despite the lack of anti-secretory activity of the peptide at these high concentrations (Figure 3.32). The PEG22 polymer alone had no effect upon Y<sub>4</sub> receptor activation (Figure 3.35).



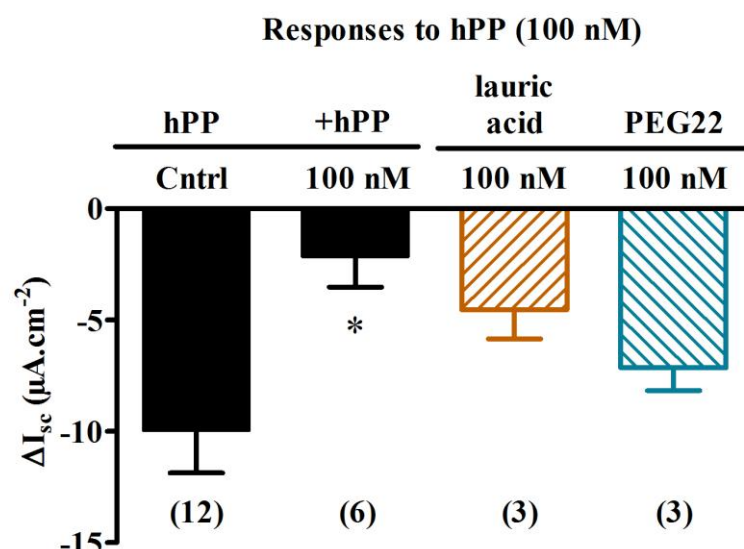
**Figure 3.35 Desensitisation of hPP activated responses following pre-treatment with increasing concentrations of [K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub> in Col-24 epithelial monolayers.** Desensitisation of hPP responses (10 nM throughout) added 45 min after increasing concentrations of [K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub>, compared with Y<sub>4</sub> desensitisation following hPP (10 nM). The PEG22 polymer alone had no effect upon Y<sub>4</sub> receptor desensitisation. Each bar represents the mean  $\pm$  1 S.E.M of pooled data with *n* numbers shown in parenthesis. Statistical differences from ‘Cntrl’ hPP responses were determined using ANOVA and Dunnett’s post-test, where \* *P* < 0.05, \*\*\* *P* < 0.001.

An analogous pattern of desensitisation with the lipidated [K<sup>22</sup>]hPP<sub>2-36</sub> variants was observed in human colon mucosa (Figure 3.36); subsequent responses to hPP (100 nM) were significantly more desensitised with the longer fatty acid conjugate, [K<sup>22</sup>(E-Pam)]hPP<sub>2-36</sub> than with all other [K<sup>22</sup>]hPP variants. An 89.0% reduction in the hPP response was observed with [K<sup>22</sup>(E-Pam)]hPP<sub>2-36</sub> compared with a 58% reduction seen with [K<sup>22</sup>(E-Capr)]hPP<sub>2-36</sub>. Unexpectedly, preliminary data indicates that [K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub> caused a 94% reduction in subsequent hPP responses compared to hPP responses in naive mucosa. This was in contrast to the resistance to desensitisation with [K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub> observed in Col-24 monolayers at the same concentration (100 nM).



**Figure 3.36 Desensitisation of hPP activated responses after pre-treatment with lipidated and PEG22 conjugated  $[K^{22}]hPP_{2-36}$  variants in human colon mucosa.** Desensitisation of hPP responses (100 nM throughout) added 60 min after the addition of  $[K^{22}(E-Capr)]hPP_{2-36}$ ,  $[K^{22}(E-Laur)]hPP_{2-36}$ ,  $[K^{22}(E-Pam)]hPP_{2-36}$  and  $[K^{22}(PEG22)]hPP_{2-36}$  (100 nM), compared with  $Y_4$  desensitisation following hPP (100 nM). Each bar represents the mean  $\pm$  1 S.E.M of pooled data with  $n$  numbers shown in parenthesis. Statistical differences from ‘Cntrl’ hPP responses were determined using ANOVA and Dunnett’s post-test, where \*  $P < 0.05$ .

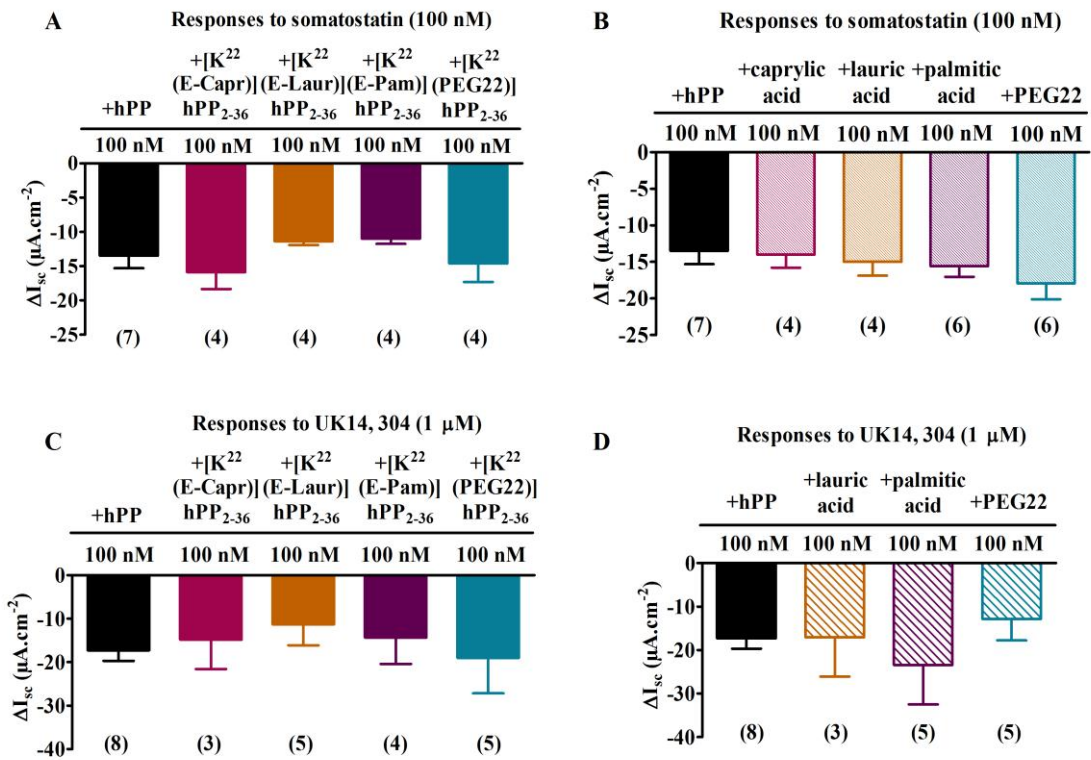
Desensitisation of the  $Y_4$  receptor was not a direct effect of the fatty acids or PEG22 polymer side chain in human colon mucosa studies (Figure 3.37). Although responses to hPP following lauric acid and PEG22 (both at 100 nM) were approximately 50 % and 25 % reduced compared with hPP in naive mucosal preparations, these responses were not statistically significant to hPP ‘cntrl’ response. The limited access to human tissue hindered further confirmation of the effects of these side chains on  $Y_4$  receptor desensitisation.



**Figure 3.37 Desensitisation of hPP activated responses following pre-treatment with lauric acid or PEG22 polymer in human colon mucosa.** Responses to hPP (100 nM throughout) added 60 min after lauric acid or PEG22 (100 nM), compared with Y<sub>4</sub> desensitisation following hPP (100 nM). Each bar represents the mean  $\pm$  1 S.E.M of pooled data with *n* numbers shown in parenthesis. Statistical differences from ‘Cntrl’ hPP responses were determined using ANOVA and Dunnett’s post-test, where \* *P* < 0.05.

### 3.6.5 Specificity of desensitisation induced by [K<sup>22</sup>]hPP<sub>2-36</sub> variants

Y<sub>4</sub> desensitisation studies revealed significant reductions in response size to hPP following the addition of lipidated [K<sup>22</sup>]hPP<sub>2-36</sub> variants. The [K<sup>22</sup>]hPP<sub>2-36</sub> variants were specific in their ability to activate G<sub>ai</sub>- coupled signalling of the Y<sub>4</sub> receptor since subsequent responses to inhibitory somatostatin (100nM) (Figure 3.38A) and UK 14,304 (1 μM) (Figure 3.38C) were not significantly different compared to control somatostatin and UK 14,304 responses in Col-24 monolayers and colon mucosa, respectively. The fatty acids and PEG22 polymer alone additionally had no significant effects upon non Y<sub>4</sub> G<sub>ai</sub>- coupled signalling in both preparations, as demonstrated in Figure 3.38B and Figure 3.38D.



**Figure 3.38 Effect of  $[K^{22}]hPP_{2-36}$  modified analogues and their respective PEG/lipid conjugates on subsequent somatostatin (100 nM) and UK 14,304 (1  $\mu$ M) responses in Col-24 monolayers and human mucosa, respectively.** Selectivity of  $G_{ai}$ -coupled signalling demonstrated by insignificant changes in the response to somatostatin following pre-treatment with (A)  $[K^{22}](E-Capr)hPP_{2-36}$ ,  $[K^{22}](E-Laur)hPP_{2-36}$ ,  $[K^{22}](E-Pam)hPP_{2-36}$  and  $[K^{22}](PEG22)hPP_{2-36}$ , and (B) their respective fatty acid or PEG polymer (all at 100 nM) in Col-24 monolayers. Responses to UK 14,304 in human colon mucosa are shown in (C) and (D). Each bar represents the mean - 1 S.E.M with  $n$  numbers shown in parenthesis. Statistical differences from control hPP responses were determined using ANOVA and Dunnett's post-test.

### 3.7 Summary of results

All lipidated variants of  $hPP_{2-36}$  were found to inhibit the elevated  $I_{sc}$  induced by VIP, revealing a slower agonist response seen previously with  $[K^{30}(E-Pam)Q^{34}]hPP$  (in Col-24 epithelial monolayers). Modification with the short chain fatty acid, E-Capr, interestingly exhibited a first phase transient profile, followed by a sustained reduction in  $I_{sc}$ . This first phase was absent from longer lipidated agonists (E-Laur and E-Pam). Therefore, the anti-secretory activity of the conjugates correlated with the length of the fatty acid chain, with the minimal requirement for possessing the enhanced prolonged monophasic anti-secretory activity being somewhere between 9 and 11 carbons atoms. Removal of the fatty acid group from the glutamate linker completely abolished the enhancement of the activity (as observed with  $[K^{22}]hPP_{2-36}$ ). Importantly, neither caprylic acid, lauric acid nor palmitic acid had any effect upon  $I_{sc}$  in both Col-24 monolayers and human tissue. Furthermore, increasing lipid length correlated with

increased latency of activation in both preparations, where  $[K^{22}(E-Pam)]hPP_{2-36}$  exhibited the slowest rate of onset. In addition to displaying protracted responses compared to  $[K^{22}]hPP_{2-36}$ , peptides conjugated to a fatty acid equal to or longer than 8 carbons retained their high potency. The extent of  $Y_4$  receptor desensitisation also positively correlated with the lipophilicity of the peptides, with palmitic acid and lauric acid conjugated peptides demonstrating significantly enhanced inhibition of further  $Y_4$  signalling compared with their non-lipidated predecessor.  $[K^{22}(E-Capr)]hPP_{2-36}$  also exhibited a concentration-dependent desensitisation profile which was more akin to that seen with the unmodified  $[K^{22}]hPP_{2-36}$  peptide, despite continued reductions in  $I_{sc}$ .

Modification of  $[K^{22}]hPP_{2-36}$  with a PEG22 polymer resulted in a complete loss of anti-secretory activity in Col-24 monolayers (100 nM) and a considerable loss in human mucosa (100 nM). The concentration response relationship (Figure 3.32) demonstrated a loss in  $Y_4$  potency and efficacy compared with the lipidated and non-modified  $[K^{22}]hPP_{2-36}$  derivatives. This is in contradiction to  $IP_3$  signalling data generated by our collaborators (Beck-Sickinger *et al.* see Appendix 1, Table 3), where the  $EC_{50}$  for the  $Y_4$  receptor was 5.3 nM. As predicted from functional data produced in Col-24 monolayers, subsequent hPP responses were unaffected in the presence of  $[K^{22}(PEG22)]hPP_{2-36}$  (1-100 nM) confirming the absence of  $Y_4$  activation. However a higher concentration of  $[K^{22}(PEG22)]hPP_{2-36}$  (3  $\mu$ M) caused significantly reduced hPP responses, despite a lack of anti-secretory response to this large peptide, and this may possibly be due to an allosteric effect on the  $Y_4$  receptor as a result of the PEG22 polymer when conjugated to the peptide at position  $K^{22}$ .

Importantly, all  $[K^{22}]hPP_{2-36}$  variants displayed high specificity towards the  $Y_4$  receptor, demonstrated by the lack of desensitisation observed with somatostatin and UK 14,304 in Col-24 monolayers and human colon mucosa, respectively.

## **4 DISCUSSION**

#### 4.1 Measurement of ion transport in colonic epithelial preparations

The present investigation utilised the heterogeneous colonic epithelial cell line, Col-24, which originates from a human adenocarcinoma (HCA-7) (Marsh *et al.*, 1993). Col-24 cells exhibit key morphological characteristics including the ability to grow on a plastic surface as a confluent polarised epithelial sheet, the existence of microvilli at the apical but not the basolateral domain and the presence of apical tight junctions. In addition, these cells have the capacity to form domes as a consequence of vectorial fluid transport in confined areas of an epithelial monolayer (Kirkland, 1985). Based on their morphology, nine subpopulations have been isolated (Marsh *et al.*, 1993) and of these, three have been identified that constitutively express  $Y_1$  &  $Y_4$  receptors; Col-1, Col-6 and Col-24. RT-PCR studies have demonstrated  $Y_4$  receptor expression in both Col-24 and Col-6 epithelia, although to a much lesser extent in the latter cell line, compared with HT-29 positive controls transfected with h $Y_4$  and h $Y_1$  receptors (Cox *et al.*, 2001b). Constitutive  $Y_4$  receptor expression was observed in Col-24 cells and were primarily selected as the most appropriate cell line to compare the anti-secretory effects of novel  $Y_4$  receptor agonists since PYY-mediated  $Y_1$  responses were absent in Col-24 cells ( $Y_1$  receptors are expressed in Col-6 cells; Tough & Cox, 1996) and secondly, nM concentrations of hPP result in transient inhibitory responses. Despite  $Y_4$  expression, the use of Col-6 epithelia in this study was not considered appropriate due the constitutive co-expression of the  $Y_1$  receptor (Tough & Cox, 1996) and the added complication of atypical  $Y_4$  receptor expression, resulting in uncharacteristic  $Y_4$ -like responses (Cox *et al.*, 2001b). Cultured epithelial cell lines have been successfully utilised to study hormone-stimulated transepithelial ion transport. Despite the fact that a large number of cell lines are derivatives of human colorectal adenocarcinomas (Fogh and Trempe, 1975; Leibovitz *et al.*, 1976), not many can maintain the polarity of their tissue of origin (Pinto *et al.*, 1983). Col-24 cells served as model epithelia in this study as they have the capability to differentiate into polarised intestinal cells and grow as a monolayer on a semi-permeable support (Cuthbert *et al.*, 1987), exhibiting a distinct brush boarder at the apical domain and the presence of tight junctions connecting neighbouring cells (Kirkland, 1985).

Although the use of a cultured epithelial cell line in this functional study offered the benefit of studying epithelial responses in the absence of neurones or other mucosal cell types, the use of more complex human mucosal preparations was invaluable, allowing the measurement of  $Y_1$ ,  $Y_2$  and  $Y_4$ -mediated anti-secretory responses (Cox and Tough,



2002; Hyland *et al.*, 2003). Immunohistochemical and functional studies have confirmed selective basolateral epithelial localisation of the Y<sub>1</sub> and Y<sub>4</sub> receptor, where their activation occurs in response to PYY and NPY (Y<sub>1</sub>) or to PP (Y<sub>4</sub>) (Mannon *et al.*, 1999; Tough *et al.*, 2006; Cox, 2007). This is in contrast to the neuronal localisation of the Y<sub>2</sub> receptor (in mouse and human), where anti-secretory responses are mediated via NPY, PYY and their shortened equivalents, NPY(3–36) and PYY(3–36) (Cox and Tough, 2002; Hyland *et al.*, 2003; Wang *et al.*, 2010). In the absence of any selective Y<sub>4</sub> receptor antagonists, the use of selective Y<sub>1</sub> and Y<sub>2</sub> receptor antagonists (BIBO3304 and BIIE0246, respectively) in human colon mucosal preparations has allowed the pharmacological dissection of the most promising analogues, and has also helped elucidate the functional significance of these peptides in controlling colonic ion secretion for the first time.

The main goal of this work was to utilise the Ussing chamber technique to evaluate the effects of PEGylation and lipidation on the behaviour of novel peptides in terms of their longevity of action, potency and efficacy. Although data generated from the Col-24 cell line does not strictly mimic that seen *in vivo*, the human origin of this cell line means that there is no interspecies variation. Therefore the Col-24 cell line can be considered comparative to human colon mucosa with respect to intestinal absorption. In addition, experiments with isolated human colon mucosa have replicated and confirmed the trends seen in the Col-24 cell line. Simultaneous biochemical measurements in COS-7 cells were performed by our collaborators within the GIPIO project; these included ligand binding and IP<sub>3</sub> signal transduction (see Appendix 1.0), which were crucial in identifying the most promising drug candidates with high receptor specificity and potency at each Y receptor. The functional data presented herein aimed to provide more biologically relevant information regarding ligand potency and efficacy with the added benefit of Y receptor antagonists to block the functional response and confirm specificity observed with the biochemical assays, hence bridging the gap between biochemical assays and *in vivo* animal studies. Furthermore, the ability to assess how the novel GIPIO peptides modulate target systems *in vitro* has also been advantageous in the early screening process, as potential side effects have also been envisaged (see section 4.7), thus facilitating hypothesis-driven *in vivo* studies.

#### **4.1.1 Electrical parameters**

In the present study, transepithelial resistance and basal  $I_{sc}$  values in Col-24 monolayers (Table 3.1) were comparable to observations seen previously with the same cell line (Cox and Tough, unpublished data) and also comparable to published data observed with Col-1 epithelial monolayers (Holliday *et al.*, 1997). In human colon mucosa, basal  $I_{sc}$  and resistance values varied (Table 3.2) but were not significantly different from previous studies (Tough & Cox 2002) with human tissue, or between the genders. Inconsistency between human colonic specimens has been observed in a number of other studies (Marrero *et al.*, 1998; Tominaga *et al.*, 1996). Marked variations have been reported in the distal colon; Marrero *et al* reported a resistance range of 52 to 220  $\Omega \cdot \text{cm}^2$  (Marrero *et al.*, 1998) following endoscopic mucosal resection from normal human volunteers. An explanation for this variability might be a divergence in the biopsy and mounting techniques employed by different groups, resulting in varying degrees of tissue damage, and a loss in tissue integrity. In comparison to the published data regarding transepithelial resistance, the data presented in this thesis exhibited error margins of less than 10 % observed between male and female mucosal preparations (Table 3.2), thus demonstrating uniformity in the dissection technique used.

#### **4.1.2 Anti-secretory Y responses are dependent on VIP stimulation**

Following  $G_s$ -coupled stimulation of VPAC<sub>1</sub> receptors by VIP,  $\text{Cl}^-$  ions exit the cell via the PKA-sensitive, apical CFTR channel, which becomes activated in response to increased intracellular cAMP (Harmar *et al.*, 2004; Banks *et al.*, 2005; see Figure 1.9). Cox *et al.* (2001b) previously reported Col-24 epithelia sensitivity to increasing VIP concentrations and subsequent analysis of pooled data generated an  $\text{EC}_{50}$  value of 2.9 nM, which was similar to that observed in Col-1 and Col-6 epithelia (Holliday *et al.*, 1997; Cox & Tough, 1995, respectively). In this thesis, increasing concentrations of VIP were used to raise the levels of anion secretion and maximally effective hPP responses were observed following 30 nM and 300 nM VIP compared with basal responses (Figure 3.2A), confirming that the response to hPP is dependent upon the secretory state of the monolayer. These results are comparable with a study performed in mouse descending colon (Tough, 2005) which demonstrated the dependence of hPP and PYY responses on the level of VIP-stimulated  $I_{sc}$ . These results are also comparable with additional previous studies in mouse descending colon (Cox *et al.*, 2001a) and rat jejunum (Cox and Cuthbert, 1988) in which responses to a single concentration of NPY were enhanced by pre-stimulation of the mucosa with increased VIP concentrations. In

either mouse or rat mucosa, the maximally effective NPY response was observed following 30 nM VIP. In addition to the enhanced NPY responses following VIP, Cox and Cuthbert (1988) found that stimulation with PGE<sub>2</sub>, forskolin, IBMX and dibutyryl cAMP also allowed enhanced NPY responses. Thus it can be concluded that G<sub>i</sub> coupled Y receptors require an accumulation of intracellular cAMP in order to exert their effects. Human mucosa preparations were not pre-treated with VIP since basal I<sub>sc</sub> levels were sufficiently high and subsequent hPP-mediated reductions in I<sub>sc</sub> were consistent between each mucosal preparation studied. This high level of basal secretion has also been reported in previous studies utilising human mucosa (Cox and Tough, 2002; Tough *et al.*, 2006), where VIP was not used to pre-stimulate the basal I<sub>sc</sub>.

#### **4.1.3 Y<sub>1</sub> and Y<sub>2</sub> receptor antagonists cause elevated I<sub>sc</sub> levels in human colon mucosae, indicative of anti-secretory tone**

The pharmacology associated with the novel analogues under investigation with the most promising *in vitro* profiles was established with the use of the competitive Y<sub>1</sub> and Y<sub>2</sub> receptor antagonists, BIBO3304 and BIIE0246, respectively. The use of these selective antagonists have been fundamental to the pharmacological characterisation of NPY, PYY, PYY(3-36) and PP, particularly in human colonic tissue co-expressing several Y receptors, and have thus allowed us to reveal the functional significance of Y<sub>1</sub> and Y<sub>2</sub> receptors in the anti-secretory response to novel Y<sub>2</sub>/Y<sub>4</sub> analogues (with potential Y<sub>1</sub> functional activity) in this study. Wieland *et al.* first demonstrated Y<sub>1</sub> competitive antagonism with BIBO3304; the Y<sub>1</sub> antagonist displayed a 1000-10000-fold lower affinity for the h/r Y<sub>2</sub>, Y<sub>4</sub> and Y<sub>5</sub> receptor, compared to subnanomolar affinity at the Y<sub>1</sub> receptor in cells stably transfected with the h/r Y<sub>1</sub> receptor or cells endogenously expressing the Y<sub>1</sub> receptor (SK-N-MC cells; Wieland *et al.*, 1998). Shortly after, Doods and co-workers revealed competitive antagonism with BIIE0246; specific binding of radiolabelled NPY was completely displaced by the Y<sub>2</sub> antagonist from hY<sub>2</sub> receptors expressed on SMS-KAN cells. Furthermore, treatment with the Y<sub>2</sub> antagonist did not compete with high concentrations of NPY (up to 1 µM) at the human Y<sub>1</sub>, Y<sub>4</sub> and Y<sub>5</sub> receptor (Doods *et al.*, 1999). Dumont and colleagues provided further evidence of competition, by demonstrating the ability of BIIE0246 to compete for specific [<sup>125</sup>I]PYY(3-36) binding sites with high affinity in HEK293 cells transfected with the rat Y<sub>2</sub> receptor, while being inactive at the Y<sub>1</sub>, Y<sub>4</sub> and Y<sub>5</sub> receptor types. In the same study, competition at the Y<sub>2</sub> receptor was also observed in rat brain and human frontal cortex membrane homogenates (Dumont *et al.*, 2000). The Y<sub>2</sub> antagonist was

subsequently used in rat hypothalamic slices to demonstrate inhibition of basal and NPY(13-36)-stimulated NPY release, revealing the pre-synaptic role of for the Y<sub>2</sub> receptor (King *et al.*, 2000). Although several other potent Y<sub>1</sub> antagonists have been developed, such as LY- 357897 (Hipskind *et al.*, 1997) and J-104870 (Kanatani *et al.*, 1999), many *in vitro* and *in vivo* studies have extensively documented the pharmacological profile of BIBO3304. The Y<sub>1</sub> receptor antagonist does not appear to induce any adverse cytotoxicity, making this compound the antagonist of choice for pharmacological studies. Furthermore, BIIE0246 is the preferred Y<sub>2</sub> receptor antagonist due to its high affinity and the large volume of data that supports its selective profile.

In this thesis, additions of both BIBO3304 and BIIE0246 to isolated preparations of human colon mucosa revealed elevated levels of I<sub>sc</sub> compared to the basal level of secretion in these specimens. Increases in I<sub>sc</sub> in response to both antagonists have previously been reported in isolated human colon mucosa (Cox and Tough, 2002; Hyland *et al.*, 2003; Tough *et al.*, 2011) and can be explained by the alleviation of endogenous inhibitory tone mediated via release of PYY and NPY. A separate study found that the BIIE0246-mediated increases in I<sub>sc</sub> were absent in mucosa from Y<sub>2</sub><sup>-/-</sup> mice (Hyland *et al.*, 2003), indicating that the responses were due to selective blockade of the Y<sub>2</sub> receptor. More recently, Tough *et al.* (2011) demonstrated the role of PYY in mediating Y<sub>1</sub> inhibitory tone, since this was absent in PYY<sup>-/-</sup> mouse mucosa. Interestingly, NPY<sup>-/-</sup> and WT tissue exhibited the same levels of Y<sub>1</sub> receptor-mediated tone (which was also reported by Hyland, 2003) and only when both peptides were ablated were Y<sub>1</sub> and Y<sub>2</sub> tonic activities lost. The authors therefore concluded that Y<sub>1</sub> anti-secretory tone is predominantly epithelial in origin and PYY-mediated; while Y<sub>2</sub> receptor-mediated tone is neuronal and mediated via both PYY and NPY, but predominantly by the latter. Furthermore, this group distinguished that Y<sub>1</sub> tone is epithelial derived, compared to Y<sub>2</sub>-mediated tone, which is neuronal in origin; however PYY released from L cells is thought to also play a role in Y<sub>2</sub> tone (Tough *et al.*, 2011). The observations presented herein have further provided evidence of tonic activation of both the Y<sub>1</sub> and Y<sub>2</sub> receptor, demonstrating the functional significance of these receptors in the gut to maintain a reduced secretory state.

#### **4.1.4 Responses to chemically modified agonist are slower in human colon mucosa compared with Col-24 monolayers**

Although pharmacological similarities exist between the two human mucosal preparations used in these studies, differences in the functional profiles to chemically modified agonists are also apparent. The most evident difference between epithelial monolayer responses and isolated colonic mucosae was that responses to all non-modified, PEGylated and lipidated peptide agonists were slower to peak in the latter preparation. With isolated mucosa experiments, the colonic segment was dissected to remove the external muscle layer and submucosa. The remaining mucosa strip was mounted in the Ussing chamber with the epithelial cell layers and underlying lamina propria still intact. The lamina propria, a vascularised region of loose connective tissue, which acts as the interface between the bloodstream and mucosal endocrine cells, may present a barrier to the basolateral additions of agonists, particularly large molecular weight molecules. In contrast, with the epithelial monolayers growing on HAWP filters, such basolateral barriers were not present and therefore the increased time-to-peak on addition of basolateral agonist is expected. The transient response profiles of non-modified peptides in Col-24 monolayers, but not in human preparations, may also be attributed to their rapid degradation by peptidases, whereas the peptidase-resistant PEGylated peptides are predicted to have increased stability and hence a more prolonged duration of action (see section 4.3.1 for further detail).

#### **4.2 Pharmacological assessment of [Q<sup>34</sup>]hPP**

Modification of hPP with a Q<sup>34</sup> substitution led to the dual Y<sub>2</sub>/Y<sub>4</sub> receptor agonist, Obinepitide ([Q<sup>34</sup>]hPP). This modification resulted in a 6-fold lower potency compared with hPP in both Col-24 monolayers and human colon mucosa. This reduction in potency with [Q<sup>34</sup>]hPP compared to the native hormone was also replicated by data generated by our collaborators (Beck-Sickinger *et al.*, see Appendix 1.0), in which IP<sub>3</sub> accumulation was measured for human Y receptors expressed in COS-7 cells. [Q<sup>34</sup>]hPP stimulated IP<sub>3</sub> accumulation with a 1.7-fold lower potency at the Y<sub>4</sub> receptor compared with hPP. In epithelial monolayers, there was no significant difference in Y<sub>4</sub> efficacy between [Q<sup>34</sup>]hPP and the native peptide. In human colon mucosa, [Q<sup>34</sup>]hPP was in fact significantly more efficacious than the native peptide, demonstrating both its Y<sub>2</sub> and Y<sub>4</sub> functional activity. An interesting observation in this study was a significantly slower time to peak with [Q<sup>34</sup>]hPP compared with native hPP in human mucosa (Figure 3.6B) but not in Col-24 monolayers. There was no significant difference in the rate of onset

within the first 5 min time point, only the time taken to reach maxima with 100 nM. A contributory factor to this latency may be the loose connective tissue in the mucosal preparations (as discussed in section 4.14), although this does not explain why responses to [Q<sup>34</sup>]hPP were slower compared with hPP. The additional Y<sub>2</sub> activation with [Q<sup>34</sup>]hPP is unlikely to contribute to the slower response, given the neuronal origin of these receptors as opposed to the epithelial derived Y<sub>4</sub> receptors. The differential response observed between the two peptides in human mucosa is yet to be determined.

#### **4.2.1 Co-stimulation of hY<sub>2</sub> and hY<sub>4</sub> by [Q<sup>34</sup>]hPP**

It is well known that the Y receptors recognise either the combined N- and C-terminal ends of the PP-fold peptides as for the hY<sub>1</sub> and hY<sub>4</sub> receptors or only the C-terminal amino acid sequence TRQRY-NH<sub>2</sub> of PYY and NPY as for the hY<sub>2</sub> receptor (Schwartz *et al.*, 1990; Beck-Sickinger *et al.*, 1994). The C-terminal sequence is also crucial for the selective Y<sub>2</sub> recognition since exchange of Q<sup>34</sup> by P<sup>34</sup> in either NPY or PYY led to a peptide that lost its hY<sub>2</sub> receptor affinity and consequently was highly selective for the hY<sub>1</sub> receptor (Fuhlendorff *et al.*, 1990). The opposite exchange in hPP, i.e. the replacement of P<sup>34</sup> with a Q<sup>34</sup> residue, led to the development of Obinepitide, a single digit nanomolar dual specific hY<sub>2</sub> and hY<sub>4</sub> receptor selective agonist (Schwartz *et al.*, 2006) as confirmed in the present study. Here, in human colon mucosa, we demonstrated functionally that the response to Obinepitide was partially inhibited (although not significantly so) in the presence of the Y<sub>2</sub> antagonist, BIIE0246 (Figure 3.8), proving the dual agonist capacity of this peptide. This was also in accordance with IP<sub>3</sub> signal transduction data whereby Y<sub>2</sub> potency for [Q<sup>34</sup>]hPP was 4000-fold higher compared to the Y<sub>2</sub> potency measured for hPP (Beck-Sickinger *et al.*, see Appendix 1.0, Table 1.0).

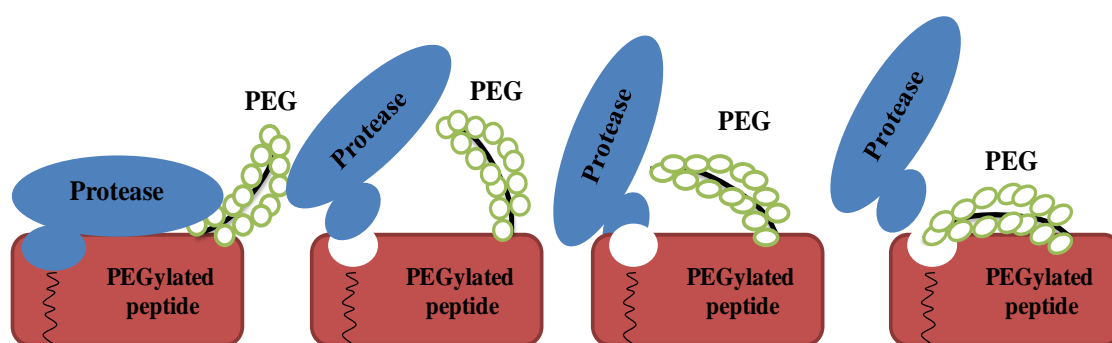
### **4.3 PEGylation of novel dual Y<sub>2</sub>/Y<sub>4</sub> and single Y<sub>4</sub> receptor agonists**

#### **4.3.1 PEGylation results in increased longevity of agonist action**

The non-PEGylated dual agonist, [K<sup>22</sup>, Q<sup>34</sup>]hPP, and the selective Y<sub>4</sub> agonist [K<sup>22</sup>]hPP<sub>2-36</sub>, both exhibited a rapid rate of onset and transient response profiles in epithelial monolayers that were akin to hPP, reaching their maximum response (with 100 nM) at approximately 5 min. Without PEG conjugation at the lysine linker, the peptide [K<sup>30</sup>, Q<sup>34</sup>]hPP was unstable (Beck-Sickinger *et al.* unpublished data) and therefore the synthesis of this unmodified peptide was not performed and not available for functional testing. The most apparent effect of PEGylation was that the *in vitro* kinetics was slower

with all PEGylated peptides in this study. PEGylation of [K<sup>30</sup>, Q<sup>34</sup>]hPP with PEG polymers of varying molecular weights (PEG2, PEG5, PEG22) resulted in prolonged agonist responses, where maxima (with 100 nM) was not reached until 45 min (Figure 3.10A). The time taken for each peptide to reach their respective maxima was faster with the smaller molecular weight PEGylated peptides and slower with the larger PEGylated peptides, and this trend was replicated in human mucosal preparations (Figure 3.11A). The PEG5- modified [K<sup>22</sup>, Q<sup>34</sup>]hPP peptides additionally demonstrated prolonged anti-secretory profiles in both Col-24 monolayers and human mucosa, again with an indication of the peak response being reached at 45 min in the former preparation (Figure 3.18A). Interestingly, PEG22 modification of both the [K<sup>22</sup>, Q<sup>34</sup>]hPP and [K<sup>22</sup>]hPP<sub>2-36</sub> variants did not reveal protracted responses, in fact there was no activity within the observation period of 45 min (discussed in more detail below, section 4.3.4). Crucially, in both colonic preparations, all PEG polymer sizes had no effect upon I<sub>sc</sub> levels, confirming that all Y<sub>4</sub>-mediated anti-secretory observations were agonist-induced and not a non-specific action of the PEG polymers.

*In vivo*, the characteristic increase in longevity of action demonstrated by the conjugation of a PEG polymer to a peptide is thought to occur via two major mechanisms; reduced clearance of the peptide via the kidneys and enhanced protection from enzymatic cleavage, together resulting in increased bioavailability. The mechanism by which PEG reduces kidney clearance is through increased hydration; two H<sub>2</sub>O molecules bind to the each ethylene glycol unit thus significantly increasing both the hydrodynamic radii by 5- to 10-fold and the molecular weight compared to their non PEGylated equivalents (Harris & Chess, 2003). *In vitro* characterisation of the PEGylated hPP variants in Col-24 monolayers and human colon mucosa demonstrated prolonged reductions in I<sub>sc</sub>; this is thought to be in part attributed to the PEG polymer shielding the peptide and reducing access to peptidases. The mechanism underlying this enzyme protection may be due to a constant movement and flexibility of the PEG polymer subsequently allowing successful interactions between the PEG-conjugated peptide and the receptor with high affinity. This model was described by Fishburn in 2007, whereby the author explained the possibility of the hydrated PEG polymer forming a barrier over the peptide domain, thus decreasing the incidence of successful collisions with the proteolytic enzyme (Fishburn, 2007), as illustrated in Figure 4.1.



**Figure 4.1 A schematic illustrating the mechanism by which a PEG polymer can provide shielding against peptide degradation.** Interaction between a plasma peptidase and a PEG-conjugated peptide is hindered in the presence of a high molecular weight PEG chain. The flexibility of the PEG polymer permits the possibility of various configurations, which may decrease the likelihood of interaction between the plasma peptidase and the target peptide. Taken from Fishburn, 2007.

### 4.3.2 Enzymatic cleavage of chemically modified PP analogues

A small number of studies have attempted to reveal the peptidases involved in the metabolism of PP; Adamo and Hazelwood (1989) were the first group to demonstrate degradation of aPP via soluble endopeptidases. Shortly afterwards, Tasaka and colleagues (1989) utilised  $^{125}\text{I}$ -PP and the acid saline extract (ASE) of rat submaxillary gland to assess peptide breakdown; ASE led to significant PP degradation, which was inhibited in the presence of a thiol protease inhibitor (Tasaka *et al.*, 1989). Although PP is a weak substrate for DPPIV compared to NPY and PYY (Mentlein *et al.*, 1993; Schon *et al.*, 1991), DPPIV cleavage has been demonstrated by Nausch *et al.* (1990).

Over the last few years, several groups have focused on establishing the stability of the native peptide and have attempted to reveal, via amino acid substitutions, which amino acid positions play a key role in stability and whether further chemical modifications can enhance the resistance to degradation. Baxter *et al.* (2010, abstract only) determined whether PP analogues with substitutions at specific positions were targeted by DPPIV and the neutral endopeptidase, NEP and whether the peptides were resistant to breakdown by both enzymes, *in vitro* and *in vivo*. With the use of high performance liquid chromatography (HPLC) and matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS), this group reported that both DPPIV and NEP were involved in the breakdown of PP. Furthermore, cleavage points were determined at position 20 and 30 when incubated with NEP *in vitro*. *In vivo*, incubation with the NEP inhibitor, phosphoramidon, enhanced reduction in food intake after two hours following a dose of 150 nmol/kg of PP. Additionally, PP plasma levels increased from 15 pmol/ml to 35 pmol/ml (at 45 min) following NEP inhibitor administration in



mice. Thus, PP analogues with targeted substitutions were shown to prevent breakdown by NEP and DPPIV, demonstrated by reduced metabolite formation *in vitro* and improved food intake inhibition in mice.

More recently, Tan *et al.* (2012) described the development of a peptidase-resistant hPP analogue (PP 1420). PP 1420 differs from hPP by five amino acid substitutions and the addition of a glycine residue at position 0. In a double-blinded, placebo-controlled randomised study, subcutaneous injection of PP 1420 (2, 4, 8 mg) to male subjects demonstrated a prolonged elimination  $t_{1/2}$  compared with the native hormone. With all doses,  $C_{max}$  was achieved one hour after dosing and then subsequently began to fall with an elimination  $t_{1/2}$  ranging from 2.4 - 2.6 hours. Given the fast elimination  $t_{1/2}$  of PP 1420, the authors predicted frequent patient dosing to maintain plasma drug levels over the course of 24 hours. Thus, this phase 1 study has not only demonstrated the successful substitution of select amino acids in the hPP sequence, thereby resulting in an extended terminal elimination  $t_{1/2}$  compared with the native hormone, but more importantly, promising tolerability in human subjects.

As previously discussed, Bellman-Sickert and colleagues demonstrated successful PEGylation of a hPP variant,  $[K^{13}, Q^{34}]hPP$  (Bellman-Sickert *et al.*, 2011). Our collaborators evaluated the stability of PEGylated N-terminally 5(6)-carboxyfluorescein-labelled  $[K^{13}, Q^{34}]hPP$  variants in human plasma against peptidase degradation. With the use of reversed phase-HPLC, fluorescence detection and MALDI-TOF-MS, this group determined that carboxyfluorescein-hPP and carboxyfluorescein- $[K^{13}, Q^{34}]hPP$  were rapidly metabolised with a  $t_{1/2}$  of around 50 hours each. Carboxyfluorescein- $[K^{13}(PEG22)Q^{34}]hPP$  demonstrated the greatest stability, with 90 % intact peptide still remaining post observation period (144 hours). A smaller PEG moiety (PEG2) also exhibited an increased resistance to proteolytic degradation, although this effect was less pronounced compared with the larger molecular weight derivative. Analysis of the degradation products of carboxyfluorescein- $[K^{13}, Q^{34}]hPP$  revealed the most unstable peptide bonds between Thr<sup>32</sup> and Arg<sup>33</sup> as well as Q<sup>34</sup> and Arg<sup>35</sup> and to a lesser extent between Leu<sup>24</sup> and Arg<sup>25</sup> as well as Arg<sup>25</sup> and Arg<sup>26</sup>. Although peptide degradation was not measured in this thesis, the encouraging data presented by Bellman-Sickert *et al.* (2011) implies increased stability with the PEGylated  $[K^{13}, Q^{34}]hPP$  variants, and this data may be used to predict increased stability with the  $[Q^{34}]hPP$  and  $[K^{22}]hPP$  variants examined in this thesis, since

prolonged anti-secretory activity was observed *in vitro* in Col-24 monolayers. It has been reported that analogues of PP in which the Met and Ala at position 30 and 22, respectively, are replaced by Lys are especially beneficial in having a longer half-life in blood plasma than native PP. Preliminary, unpublished data generated within the GIPIO collaboration has demonstrated protease resistance by incubation of 5(6)-carboxyfluorescein-labelled [K<sup>22</sup>, Q<sup>34</sup>]hPP and the corresponding PEGylated analogue with porcine liver extract homogenates. In keeping with the prolonged reductions in I<sub>sc</sub> observed in this thesis to PEGylated peptides, a 20-fold greater half life was seen for the PEG5ylated [K<sup>22</sup>, Q<sup>34</sup>]hPP variants (Made *et al.*, unpublished data). Interestingly, an increased stability was also reported for [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP, despite the lack of functional activity seen in both Col-24 monolayers and human colon mucosa. This may be due to the large PEG chain sterically hindering the receptor binding site in an attempt to block exposure to proteolytic destruction (Figure 4.1).

The instability of Y receptor peptide agonists against proteolytic enzymes is the most discouraging factor for their therapeutic use as new treatment modalities. Although the short metabolic stability of the native peptides can be compensated by continuous *i.v.* infusion, this does not fundamentally overcome the effect of rapid degradation via proteolytic enzymes. Thus, the development of a prolonged-acting form of a Y<sub>2</sub>, Y<sub>4</sub> or Y<sub>2</sub>/Y<sub>4</sub> agonist *in vivo* has been the focus of much recent research. While the effectiveness of PEGylation in enhancing the stability of peptide pharmaceuticals has been validated for years, the underlying mechanism remained poorly understood until recently. The published data described above have helped to refine the mechanism behind the increased stability and prolonged action, corroborating the data presented in this thesis.

#### **4.3.3 PEGylation results in a latency in response activation**

Interestingly, all PEGylated peptides in this study displayed latency in onset, with larger PEG-peptide sizes inducing a greater degree of delay (as observed in epithelial monolayers and human mucosa). This delay in onset may be a result of a slower rate of peptide-receptor association and a number of factors could be responsible for the reduction in apparent activation rates of PEGylated peptides. Very few studies have been published regarding PEGylated agonist-GPCR molecular interactions and many have been largely based on PEGylated antibodies. Kubetzko *et al.* (2005) studied the PEGylation of an antibody construct and published two mechanisms that may be

involved; intra-molecular and inter-molecular blocking. With the former, the PEG moiety is thought to directly obstruct the antibody's binding region, and with the latter, the PEG polymer could deter binding of the antibody to adjacent sites in the bound conformation. With the use of binding assays on HER-2-overexpressing cells and mass-transport limited BIAcore measurements, Kubetzko *et al.* (2005) demonstrated that over 90% of the PEGylated antibody constructs in solution exist in an intra-molecularly blocked conformation (with the PEG moiety directly obstructing the antibody's binding region). This theory may in part explain why PEGylated molecules exhibit low immunogenicity and high resistance to proteolytic destruction *in vivo*, since they are predominately shielded by the PEG moiety itself. Nevertheless, the favourable characteristics of PEGylation may still be achieved with strategies such as short or branched PEGylation, or even with more novel approaches such as reversible PEGylation (Peleg-Shulman *et al.*, 2004) to avoid loss in therapeutic activity.

In terms of the PEGylated peptides assessed in this study, there is a clear correlation between the molecular weight of the PEG polymer and the degree of latency in activation with both the  $[K^{30}, Q^{34}]hPP$  and  $[K^{22}, Q^{34}]hPP$  variants in Col-24 monolayers (Figure 3.10B and Figure 3.18B). This is suggestive of the intra-molecular blocking described above (Kubetzko *et al.*, 2005) whereby PEGylation at these sites is likely to sterically hinder  $Y_4$  receptor association by the PEG itself. This is in accordance with the lack of functional output seen with the larger PEG-modified peptides,  $[K^{22}(PEG22)Q^{34}]hPP$  and  $[K^{22}(PEG22)]hPP_{2-36}$ . It is predicted that the delayed rate of onset observed in Col-24 monolayers is a result of slow association of the peptide to the binding target, since the delayed rate of onset was not observed with the non-PEGylated counterparts. Although association rates were not determined directly, this is the most likely hypothesis.

#### **4.3.4 The effect of PEGylation on peptide efficacy and potency**

Although a PEG polymer has the capacity to enhance the overall bioavailability of a peptide, this is often detrimental to the receptor binding affinity, as a consequence of steric hindrance. Thus, although the equilibrium between the pharmacokinetic and pharmacodynamic properties is altered in the presence of the PEG side chain, the improved efficacy coupled with the potential for reduced patient dosing outweigh the disadvantage of a loss in binding affinity. The main objective of early PEGylation attempts was to enhance drug exposure and minimise the risk of adverse events,

however little attention was paid to conserving receptor potency. Recently, more consideration has gone into maximising drug exposure, whilst retaining drug potency. Unlike the transient responses to hPP and all other non-modified peptides, the PEGylated peptides demonstrated a delay in the rate of onset, followed by sustained reductions in  $I_{sc}$ . To construct concentration-response curves and make an assessment of potency, responses to each concentration of PEGylated peptide were taken at the maxima produced and in most cases, this was at 45 min. The resultant concentration-response curves for the PEG-modified  $[K^{30}, Q^{34}]hPP$  series of peptides (Figure 3.14) illustrate that peptide modification with a PEG2 polymer generated a maxima that was only marginally greater (and not significantly so) than that of hPP but was significantly smaller than that of  $[K^{30}(PEG5)Q^{34}]hPP$ , despite the prolonged anti-secretory response. Modification of  $[K^{30}, Q^{34}]hPP$  with a 5 kDa PEG thus resulted in increased longevity of action coupled with superior efficacy compared with its non-PEGylated counterpart  $[Q^{34}]hPP$ . Interestingly, the maximum observed for  $[K^{30}(PEG5)Q^{34}]hPP$  was also greater (again, not significantly so) than that observed with  $[K^{30}(PEG22)Q^{34}]hPP$ , highlighting the role that PEG molecular weight above a threshold concentration may play in controlling the interaction at the receptor. In this case, the mechanism of inter-molecular blocking may be involved, where the PEG tail may be hindering other receptor sites in the vicinity and reducing the feasibility of successful peptide-receptor interactions. This is a likely possibility for the  $Y_4$  receptor, since evidence indicates close proximity (receptor homodimerisation) in plasma membrane microdomains. Homodimerisation of the rhesus (rh)  $Y_4$  receptor was first reported by Berglund and co-workers (Berglund *et al.*, 2003b); each rhY receptor was fused to RLUC at the carboxy termini of the receptor and a green fluorescent protein (GFP) was subsequently fused to rhY<sub>4</sub> receptor. Using BRET2 technology, Berglund *et al.* established that the rhY<sub>4</sub> receptor exists as a dimer prior to stimulation at the cell surface of HEK293 cells, and then dissociates following agonist binding. High BRET2 ratios, indicative of a close association of RLUC-tagged rhY<sub>4</sub> receptors and GFP, also confirmed Y<sub>4</sub>-specific homodimerisation. High cell expression levels for the rhY<sub>4</sub>-GFP were observed in this study. Since it has been reported that recombinant protein over-expression in cells can encourage receptors to come together in an atypical manner (Devi, 2001), Berglund *et al.* validated their findings by demonstrating no significant difference between the expression levels of the RLUC-tagged rhY<sub>4</sub> receptor and the RLUC-tagged human opioid  $\delta$  and  $\mu$  receptors in addition to the rhY<sub>2</sub> receptor, thus providing confirmation that the homodimerisation was a particular characteristic of the rhY<sub>4</sub> receptor and not a

consequence of protein over-expression. In addition to these findings, Berglund *et al.* also reported a lack of rhY<sub>4</sub> receptor heterodimerisation with other members of the Y receptor family; aside from dimerisation with itself, the rhY<sub>4</sub> receptor interestingly displayed a high degree of interaction with the Y<sub>2</sub> receptor. High sequence homology is thought to play a role in determining heterodimerisation between receptors (Ramsay *et al.*, 2002); interestingly, the Y<sub>2</sub> and Y<sub>4</sub> receptors share a much lower sequence identity compared with the Y<sub>1</sub> and Y<sub>4</sub> receptor, however the lowest BRET2 ratio for heterodimerisation was unexpectedly observed between the latter pair of receptors. In this thesis, given the lower efficacy observed for the PEG22 conjugated [K<sup>30</sup>, Q<sup>34</sup>]hPP peptide, and the fact that the Y<sub>4</sub> receptor can exist as a homodimer, it is likely that the PEG tail may be inter-molecularly interacting with adjacent unbound Y<sub>4</sub> receptors within the dimer, significantly hindering further binding.

In terms of potency, the [K<sup>30</sup>, Q<sup>34</sup>]hPP series of peptides demonstrated slightly increased potencies relative to [Q<sup>34</sup>]hPP (EC<sub>50</sub> values were 4.3, 13.8, and 4.3 nM for [K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP, [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP and [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP, respectively, compared with 36.0 nM for [Q<sup>34</sup>]hPP) in Col-24 monolayers. This retention in Y<sub>4</sub> potency was in accordance with the IP<sub>3</sub> data generated by our collaborators, where all PEGylated peptides exhibited nM Y<sub>4</sub> receptor potencies (see Appendix 1.0, Table 1). A K<sup>22</sup> modification interestingly resulted in differential effects upon Y<sub>4</sub> signalling efficacy. Despite the prolonged anti-secretory responses seen with [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP, there was no significant increase in the maxima compared with the non-PEGylated [K<sup>22</sup>, Q<sup>34</sup>]hPP or hPP, albeit at a later time point (Figure 3.20). Nevertheless, [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP did exhibit a 10-fold higher potency compared with the unmodified counterpart. Surprisingly, modification of [K<sup>22</sup>, Q<sup>34</sup>]hPP with a PEG22 polymer was in fact unfavourable in terms of efficacy and potency; [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP was inactive across the concentration range of 0.3 and 1000 nM in contrast with observations with [K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP in Col-24. Recent data with [K<sup>13</sup>(PEG20)Q<sup>34</sup>]hPP showed that it had reduced affinity for the Y<sub>4</sub> receptor (Bellman-Sickert *et al.*, 2011). Interestingly, IP<sub>3</sub> data generated by our collaborators indicates only a small reduction in Y<sub>4</sub> potency with [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP (14.0 nM) compared with the non PEGylated [K<sup>22</sup>, Q<sup>34</sup>]hPP (0.5 nM). This loss in Y<sub>4</sub> efficacy and potency was also observed with [K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub> (Figure 3.32), despite demonstrating nM affinity for the Y<sub>4</sub> receptor in IP<sub>3</sub> accumulation studies (see Appendix 1.0, Table 2). This difference could be attributed to the difference in time points when measuring potency in both assays. The measurement of IP<sub>3</sub>

production is taken at 5 min after agonist addition since IP<sub>3</sub> levels typically reach a peak within a few seconds. In the functional assay with Col-24 monolayers, due to the latency in the rate of onset of an anti-secretory response, measurements of the maximum reduction in I<sub>sc</sub> are not measured until approximately 45 min, when steady state is reached.

The enhanced and prolonged responses to PEGylated peptides have been described previously *in vivo* (DeCarr *et al.*, 2007). In lean C57BL/6 mice, administration of PYY(3-36) (2.5 µmol/kg) induced a considerable (42%) reduction in food intake compared with control mice at a 4 hour time point. Twenty four hours after the administration of PYY(3-36), a significant 9% reduction was still observed. The PEG5ylated PYY(3-36) variant (PEG5-PYY(24-36)-L<sup>31</sup>) resulted in a loss in efficacy compared with the non-modified PYY(3-36), where 5.6 µmol/kg induced a 22% reduction in food intake at 4 hours and minimal reduction at 24 hours. No activity was seen with the peptide at 4 hours when the PEG size was increased to 40 kDa (PEG40-PYY(24-36)-L<sup>31</sup>), however a small reduction of 12% and 8% was measured at 24 and 48 hours, respectively. Therefore modification of the native peptide with a 5 or 40 kDa PEG polymer did not lead to a significant improvement in efficacy compared with PYY(3-36). Interestingly, administration of PEG20-PYY(24-36)-L<sup>31</sup> at the same concentration induced a similar reduction in food intake to PYY(3-36) (42 % at 4 hours), although contrary to that seen with PYY(3-36), the result remained statistically significant at 24 and 46 hours. Therefore significant reductions in food intake were reported 24 and 48 h subsequent to the administration of PEG20 and PEG40-conjugated PYY(3-36) variants, suggesting an increased longevity of action in comparison to PYY(3-36) itself. Interestingly, the largest PEG modification (PEG40-PYY(24-36)-L<sup>31</sup>) did not elicit a response within the 4 hour observation period, thus corroborating findings in this thesis that larger PEG-conjugated peptides display a latency in the rate of onset, suggestive of the intra-molecular blocking described above (Kubetzko *et al.*, 2005).

The preservation of potency observed in this thesis between PEGylated and non-PEGylated peptides corroborates findings reported by Lumb *et al.* (2007) who described a Y<sub>2</sub> selective PEGylated peptide and its capacity to decrease food consumption in both lean and diet induced obese mice. Peptide 1, consisting of the 25-36 amino acid core sequence of hPYY conjugated to an N terminally positioned 2-mercaptosuccinic acid,

displayed superior Y<sub>2</sub> receptor affinity *in vitro*. In order to achieve enhanced efficacy *in vivo*, the amine of the N-terminal modifying group was changed to a thiol moiety suitable for site-specific modification with a PEG polymer. Replacement of the methylmercapto moiety with a thiol followed by PEGylation via a maleimide group either with the linear PEG structure of 5 or 20 kDa or with a branched 40 kDa PEG polymer resulted in analogues that exhibited *in vitro* activity at the Y<sub>2</sub> receptor comparable to that of the non-PEGylated analogue. The authors concluded that in general, PEG size or branching does not have a significant effect on *in vitro* Y<sub>2</sub> receptor binding or activation, with the EC<sub>50</sub> and K<sub>i</sub> values at the Y<sub>2</sub> receptor within 2-fold of the values of the non-PEGylated peptides.

The data presented in this thesis regarding peptide potency is in partial agreement with finding reported by Lumb *et al.* (2007). Although this group have demonstrated a conservation in potency with PEGylated PYY analogues with molecular weights of 5, 20 and 40 kDa, both hPP analogues [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP and [K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub> in this study exhibited a significant loss in Y<sub>4</sub> potency. PEG22-ylation at position 22 of the PP sequence is therefore detrimental to potency and efficacy, as determined by functional anti-secretory activity in both colonic preparations. The evidence presented herein warrants investigation into the clinical significance of the increased stability of PEGylated hPP analogues *in vivo*.

#### 4.4 Effect of PEGylation on Y<sub>4</sub> receptor desensitisation

Having established the pharmacology associated with the chemically modified [K<sup>30</sup>, Q<sup>34</sup>]hPP, [K<sup>22</sup>, Q<sup>34</sup>]hPP and [K<sup>22</sup>]hPP<sub>2-36</sub> derivatives, the ability of PEGylated variants to induce desensitisation to a subsequent response to hPP was assessed. The transient nature of hPP responses is indicative of rapid Y<sub>4</sub> receptor desensitisation in the presence of an agonist, and is comparable to that seen with epithelial Y<sub>1</sub> receptors (Holliday *et al.*, 2005). Early studies performed by Voisin *et al.* reported an inability of the Y<sub>4</sub> receptor to desensitise in CHO cells, but this was possibly due to the 24 hour exposure to PP and also a failure to measure degradation of the agonist (Voisin *et al.*, 2000). The same group also reported the inability of hY<sub>4</sub> receptors tagged at the C terminus to redistribute from the plasma membrane after 24 h pre-treatment with hPP, however this was contradictory to findings reported by Parker *et al.* (2001 and 2002) whereby it was shown that [<sup>125</sup>I]PP was effectively and rapidly sequestered from the plasma membrane into endosomes by rat Y<sub>4</sub> receptors in the same cell line. Using BRET, Berglund *et al.*

demonstrated recruitment of  $\beta$ -arrestin 2 following  $Y_4$  receptor activation with hPP. In this thesis, hPP (3-300 nM) reduced VIP stimulated  $I_{sc}$  in a concentration-dependent manner in Col-24 monolayers (Figure 3.4). Following the initial response to 10 nM hPP, responses to a subsequent second addition of hPP (10 nM) were abolished due to homologous desensitisation. Homologous and cross-desensitisation of constitutively expressed  $Y_4$  receptors has been demonstrated previously in human colonic epithelial monolayers using species variant PPs (Cox *et al.*, 2001b).

The literature regarding  $Y_2$  receptor internalisation is controversial. It has been reported that this  $Y$  receptor type internalises slowly (Berglund *et al.*, 2003; Gicquiaux *et al.*, 2002; Ouedraogo *et al.*, 2008). In contrast, other studies have demonstrated rapid internalisation of the  $Y_2$  receptor after agonist treatment in HEK293 cells, with a rate comparable with the h $Y_1$  and h $Y_4$  receptors (Holliday *et al.*, 2005; Bohme *et al.*, 2008; Kilpatrick *et al.*, 2010). Recently, Walther *et al.* (2010) confirmed with the use of fluorescence microscopy that h $Y_2$  receptor internalisation was not limited to HEK293 cells; internalisation was measured in two human cell lines known to express the receptor endogenously, SMS-KAN and MHH-NB-11. Walther *et al.* demonstrated a comparable mode of internalisation in all three cell lines and could therefore conclude that h $Y_2$  receptor internalisation was not dependent on the cell type but that it occurred in cells that endogenously express the receptor. This group revealed rapid arrestin-3 recruitment to the plasma membrane upon h $Y_2$ R stimulation in all 3 cell lines tested. Quantification of receptor internalisation in the presence or absence of arrestin-3 over-expression by cell surface ELISA, as well as analysis of h $Y_2$ R/arrestin-3 interaction by BRET, strongly suggested an arrestin-3-dependent internalisation process, consistent with the data obtained by fluorescence microscopy. Thus, Walther *et al.* published the first direct demonstration of h $Y_2$ R interaction with arrestin-3 and this was in good agreement with BRET2 studies showing slow association rates of the rhesus  $Y_2$ R with arrestin-3 in HEK293 cells (Berglund *et al.*, 2003).

With the PEGylated [ $K^{30}$ ,  $Q^{34}$ ] derivatives,  $Y_4$  desensitisation studies revealed unexpected differences that indicated an apparent lack of desensitisation following [ $K^{30}$ (PEG5) $Q^{34}$ ]hPP; no significant  $Y_4$  desensitisation occurred in the presence of 0.3 – 1000 nM of the peptide. This lack of desensitisation to subsequent hPP responses was also observed following [ $K^{30}$ (PEG2) $Q^{34}$ ]hPP (1-1000 nM), however a significant reduction in the hPP response was recorded with 30 nM peptide. Increasing the PEG



size ( $[K^{30}(PEG22)Q^{34}]hPP$ ) resulted in partial inhibition of hPP responses between 0.1 and 1000 nM and this reduction in the size of the hPP response was also observed in human colon mucosa. An analogous pattern of desensitisation was also observed for the  $[K^{22}, Q^{34}]hPP$  series of peptides; pre-treatment with  $[K^{22}(PEG5)Q^{34}]hPP$  (0.1-100 nM) did not induce  $Y_4$  receptor desensitisation in Col-24 epithelia (Figure 3.21B), whereas significant hPP desensitisation occurred in the presence of  $[K^{22}(PEG22)Q^{34}]hPP$  (0.3-1000 nM, but not 100 nM). This observation was surprising since the PEG22-peptide did not exhibit a measurable anti-secretory response in both Col-24 monolayers and human colon mucosa. All PEGylated peptides within this study demonstrated differential profiles in terms of efficacy. Both PEG5-ylated peptides demonstrated high efficacy (though this was much greater with  $[K^{30}(PEG5)Q^{34}]hPP$  compared with  $[K^{30}(PEG5)Q^{34}]hPP$ ), whereas  $[K^{30}(PEG2)Q^{34}]hPP$  and  $[K^{30}(PEG22)Q^{34}]hPP$  exhibited lower efficacies and  $[K^{22}(PEG22)Q^{34}]hPP$  was inactive.

Up until the last two decades, ligand-mediated activation of GPCRs and downstream signalling events were thought to be a linear process; this theory explained that one GPCR could be activated by a number of agonists which could then induce one distinct stable conformation. It is this one conformation which was thought to permit the coupling of one G-protein subtype, resulting in the same intracellular responses mediated by stimulation or inhibition of the receptor. A number of studies have recently suggested that the efficacy of an agonist is not always indicative of observations seen in experimental data, leading to the several novel theories that may help predict a more accurate response with a given agonist. These theories suggest that a receptor can employ a number of diverse active conformations once activated and stabilised by an agonist (Kenakin, 2007), leading to the stimulation of distinct effectors corresponding to that receptor (Urban *et al.*, 2007; Violin and Lefkowitz, 2007). An expansion of this model is that various agonists for one receptor subtype could also induce differential mechanisms of desensitisation, since one distinct receptor conformation may acquire enhanced or reduced affinity for regulatory proteins such as GRKs or arrestin.

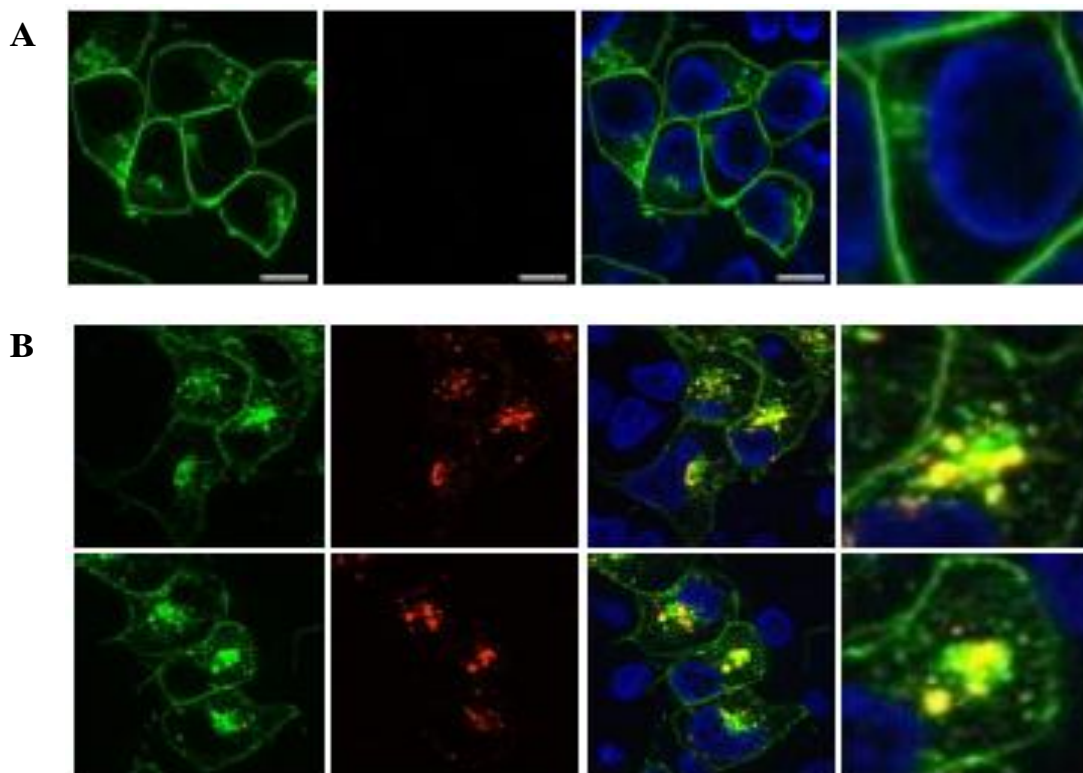
Studies with the  $\beta_2$ -AR have been pivotal in establishing ligand-directed signal specificity. In 2008, Drake *et al.* demonstrated that a range of  $\beta_2$ -AR ligands displayed considerable bias towards  $\beta$ -arrestin signalling. Despite their low efficacy for G-protein-dependent signalling, each ligand was capable of stimulating  $\beta$ -arrestin translocation to a much greater degree relative to their efficacy (Drake *et al.*, 2008). It was subsequently

determined that biased ligands for  $\beta$ -arrestin collectively shared a common structural feature, promoting the idea that ligand-receptor complexes can adopt stable conformations which encourage recruitment of  $\beta$ -arrestins. More recently, the use of biophysical techniques, such as intra-molecular FRET (fluorescent resonance energy transfer) have allowed the comprehensive real-time examination of active receptors. In 2009, Zurn *et al.* utilised a C-terminally labelled  $\alpha_2$ -AR with CFP (cyan fluorescent protein) and introduction of a fluorescein arsenical hairpin binder, targeted to different sites in the third intracellular loop of the  $\alpha_2$ -AR, to demonstrate that a number of receptor rearrangements occur upon binding to both partial and full agonist, revealed by differences in FRET efficiencies (Zurn *et al.*, 2009).

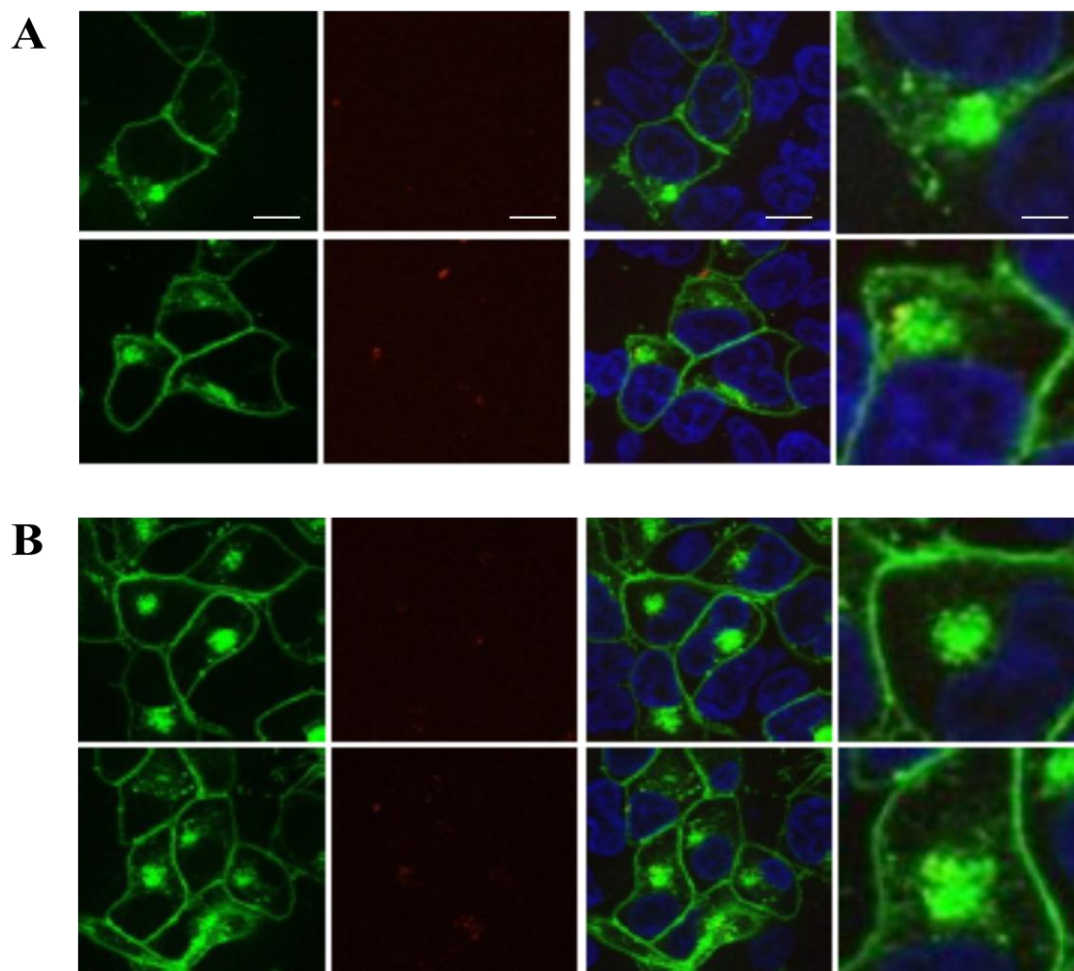
Agonist-mediated signal specificity has also been well documented with the opioid receptors; unlike the  $\mu$ -opioid receptor agonists etorphine and [D-Ala<sup>2</sup>,N-MePhe<sup>4</sup>, Gly-ol]enkephalin (DAMGO), which can promote recruitment of arrestin to the  $\mu$ -opioid receptors and induce rapid internalisation, the  $\mu$ -opioid agonist, morphine, cannot (Keith *et al.*, 1996). It was once thought that the paradoxical feature of morphine to induce little desensitisation despite the ability to stimulate receptor signalling was a consequence of transient receptor binding, resulting in activation but not phosphorylation of the receptor. At the time, further supporting data was provided by Zhang *et al* who demonstrated morphine to act as a partial agonist (Zhang *et al.*, 1998), thus providing another explanation for morphine's ineffectiveness in stimulating GRK-mediated phosphorylation and arrestin recruitment. An additional study further corroborated these findings utilising HEK-293 cells over-expressing GRK2. Over-expression of the regulatory protein resulted in arrestin translocation to the  $\mu$ -opioid receptor and internalisation (Bohn *et al.*, 2004). However more recent studies have challenged the initial findings and have demonstrated full agonism at the  $\mu$ -opioid receptor, despite the lack of ability to promote arrestin recruitment and internalisation, and also the existence of GRK2 over-expression (Groer *et al.*, 2007; Xu *et al.*, 2007). Johnson *et al.* went on to demonstrate that both morphine and DAMGO induced rapid desensitisation of a K<sup>+</sup> current via different mechanisms in HEK293 cells expressing a G protein-coupled inwardly rectifying K<sup>+</sup> channel (Johnson *et al.*, 2006). The addition of a PKC inhibitor led to a reduction in morphine-mediated desensitisation, although the same was not observed with DAMGO; desensitisation was only observed in the presence of a dominant negative mutant form of GRK2 when co-transfected. From these findings it was concluded that both morphine and DAMGO are able to exert differential

stabilisation effects upon the  $\mu$ -opioid receptor by coupling to G proteins and activating the inwardly rectifying K<sup>+</sup> channel, but possessing a varying affinity for GRK2 and PKC. Several studies have now shown that a decline in ligand-induced signalling is not always indicative of the ligand's ability to desensitise its receptor. The data described above suggests that regulation of receptor activation and subsequent receptor phosphorylation is in part dependent on the ligand itself, thus opening the possibility of developing pharmaceuticals that are highly efficacious but do not result in tolerance.

A further and more likely explanation for the lack of desensitisation observed with the PEG5-ylated peptides is that the process of internalisation is disrupted due to the presence of the PEG polymer. This hypothesis has been verified by our collaborators with the use of stably transfected HEK-293 cells with an enhanced YFP-tagged Y<sub>4</sub> receptor and co-localisation with TAMRA-(5(6)-carboxytetramethylrhodamine) peptides (Figure 4.2A). With 100 nM TAMRA-hPP, Mäde *et al* (unpublished) demonstrated fast and specific internalisation of EYFP-tagged Y<sub>4</sub> receptor and the unmodified ligand in co-localisation experiments (Figure 4.2B; for full experimental methods, see Appendix 1.0). Although internalisation studies are yet to be performed on the [K<sup>30</sup>, Q<sup>34</sup>]hPP series of peptides, these studies have been performed with the [K<sup>22</sup>, Q<sup>34</sup>]hPP series. Interestingly, TAMRA modified [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP (100 nM) and [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP (1  $\mu$ M) demonstrated impaired cellular uptake (Figure 4.3) relative to hPP under the same conditions. Thus, it seems the EYFP-hY<sub>4</sub>R internalised more slowly when incubated with a PEGylated peptide conjugate, with the peptide possibly anchored within the cell membrane. This effect was contrary to the fast and specific internalisation observed for hPP and also appears to be dependent on PEG size, since [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP demonstrated poor internalisation at 30 min and very little internalisation at 60 min.

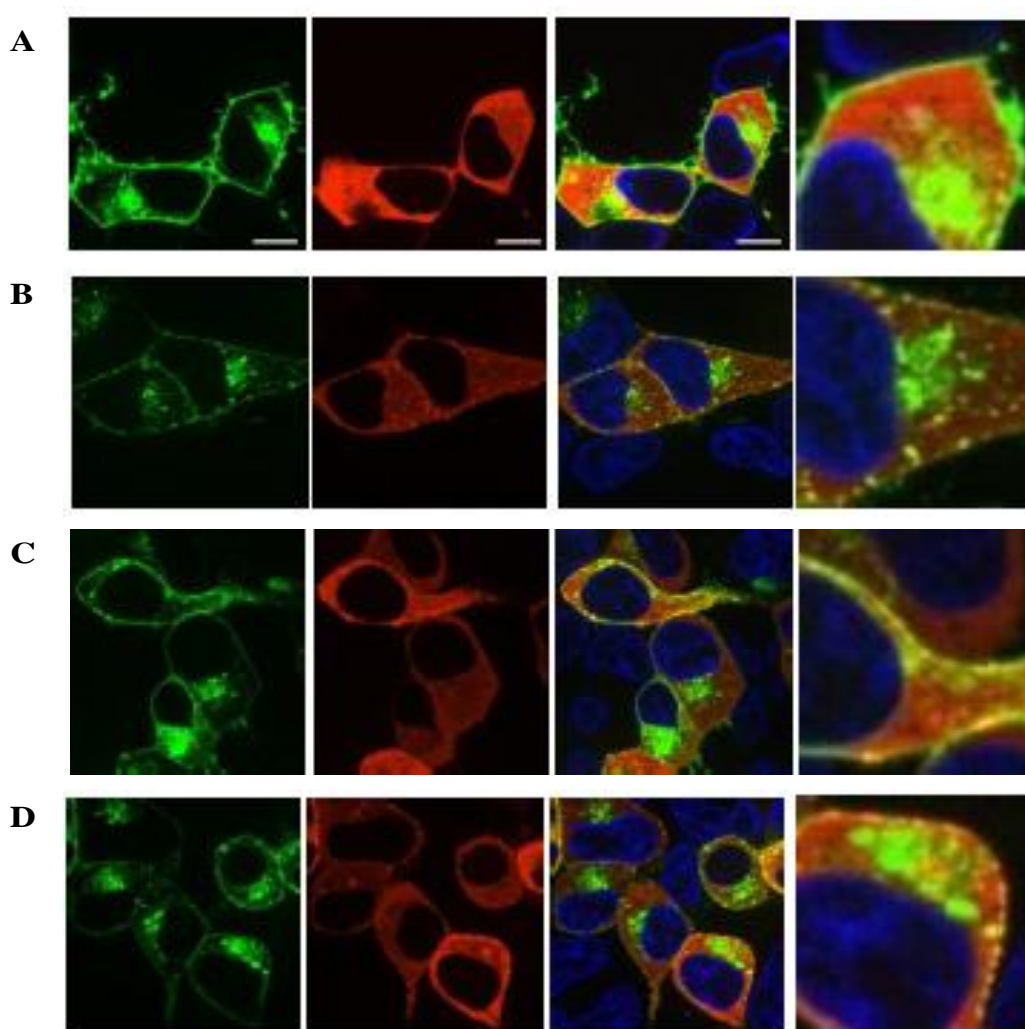


**Figure 4.2 Localisation of hY<sub>4</sub> receptor C-terminally fused to EYFP in HEK293.** (A) No agonist; hY<sub>4</sub>R-EYFP prior to stimulation of the receptor shown in green, TAMRA-modified peptide shown in red with nuclei visualised with Hoechst33342 shown in blue, and a 3 x magnification of a chosen cell is shown at the far right. The scale bar represents 10  $\mu$ m. (B) Stimulation with unmodified peptide (hPP, 100 nM) elicits rapid internalisation of the receptor-peptide complex (hY<sub>4</sub>R-EYFP and peptide co-localisation is shown in yellow). The upper and lower images of (B) demonstrate ligand and receptor localisation at 30 and 60 min, respectively.



**Figure 4.3 Stimulation of HEK-293 cells expressing hY<sub>4</sub>R-EYFP co-localised with PEGylated peptides.** hY<sub>4</sub>R-EYFP is shown in green, TAMRA-modified peptide shown in red with nuclei visualised with Hoechst33342 shown in blue. A 3 x magnification is shown at the far right, with scale bars representing 10 μm. (A) Stimulation with 100 nM [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP and (B) stimulation with 1 μM [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP results in weaker internalisation of the receptor-peptide complex, than that observed after hPP under equivalent conditions (Figure 4.2). The upper and lower images here demonstrate ligand and receptor localisation at 30 and 60 min, respectively.

To address the impact of arrestin on the internalisation process, co-transfection of arr-3-mCherry with hY<sub>4</sub>R-EYFP was performed in HEK-293 cells and again analysed by fluorescence microscopy. Prior to agonist treatment receptor- EYFP fluorescence was mainly localised in the plasma membrane and arr-3-Cherry fluorescence was homogenously distributed in the cytoplasm. hPP (100 nM) stimulation led to a rapid redistribution of arr-3-mCherry from a diffuse cytoplasmic to a membrane-associated vesicular pattern only for the unmodified peptide. For the PEGylated peptides, no arr-3-mCherry translocation was detected, as indicated by the sustained diffuse distribution of red fluorescence throughout the cytoplasm after PEGylated ligand exposure (Figure 4.4).



**Figure 4.4 Time-dependent arrestin3-promoted hY<sub>4</sub>R internalisation upon stimulation with hPP, [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP and [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP.** (A) hY<sub>4</sub>R-EYFP and arrestin-3 localisation prior to stimulation, with the receptor shown in green, arr-mCherry shown in red, with nuclei visualised with Hoechst33342 shown in blue and a 3 x magnification (far right-hand images). The scale bar represents 10 μm throughout. Redistribution of arr-mCherry in response to hPP 100 nM (B), [K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP 100 nM (C) and [K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP 1 μM (D), all captured at 15 min.

The fact that the PEGylated peptides,  $[K^{30}(PEG2)Q^{34}]hPP$ ,  $[K^{30}(PEG5)Q^{34}]hPP$  and  $[K^{22}(PEG5)Q^{34}]hPP$ , did not induce  $Y_4$  receptor desensitisation in Col-24 monolayers and human colon mucosa was complimented by the lack of arrestin-3 recruitment to the receptor ( $[K^{22}(PEG5)Q^{34}]hPP$  only, Figure 4.4C).  $[K^{30}(PEG22)Q^{34}]hPP$  however did partially reduce further  $Y_4$  signalling (as demonstrated by subsequent reduced hPP responses), even without the presence of arrestin in HEK-293 cells, demonstrated by our collaborators (Figure 4.4D). There could be several reasons for this; the large PEG22 polymer conjugated to the peptide may interact with the receptor in such a way as to prohibit the formation of the receptor state that recruits  $\beta$ -arrestin. Another possibility is that the reduced responses to hPP after pre-treatment with  $[K^{30}(PEG22)Q^{34}]hPP$  and  $[K^{22}(PEG22)Q^{34}]hPP$  may not be due to desensitisation of the receptor at all, but allosteric hindrance of the receptor binding site caused by the large PEG side chain, blocking subsequent binding. Furthermore, we cannot exclude the possibility that there may be a transient interaction of the  $Y_4$  receptor-peptide complex with  $\beta$ -arrestin that is not detected within the time frame of the imaging experiments with the HEK293 cells. The loss of hPP responses and the lack of arrestin recruitment suggests that  $[K^{30}(PEG22)Q^{34}]hPP$  may mediate GPCR internalisation in an arrestin-independent manner, however as shown in Figure 4.3, the peptide is also not internalised and thus the former explanations are more plausible. PEG22-ylation of the  $[K^{22}]hPP_{2-36}$  peptide interestingly did not mimic the full desensitisation pattern seen with  $[K^{30}(PEG22)Q^{34}]hPP$ , but was analogous to the partial agonism and partial desensitisation observed with  $[K^{30}(PEG2)Q^{34}]hPP$  and  $[K^{30}(PEG5)Q^{34}]hPP$ , demonstrating a lack of hPP desensitisation with concentration range of 1-100 nM in Col-24 monolayers (Figure 3.29). A concentration of 1  $\mu$ M however, did result in a significant loss in subsequent hPP responses and at 3  $\mu$ M  $[K^{22}(PEG22)]hPP_{2-36}$  even more so, despite the low efficacy of this PEG-22 modified  $[K^{22}]hPP_{2-36}$  analogue in both colonic preparations at these high concentrations (Figure 3.26).

The ability of PEGylated analogues to disrupt the process of internalisation has been reported previously. In 2010, Sen *et al.* PEGylated the recombinant coagulation factor VIIa (rFVIIa), which is widely used for treatment of haemophilia, and is cleared from the circulation rapidly with a plasma half-life of 2–4 hours. This group investigated the effect of  $^{125}I$ -labelled rFVIIa and glycoPEGylated rFVIIa on binding to its cellular receptors and its subsequent internalisation in endothelial cells and fibroblasts. Modification of rFVIIa with PEG impaired rFVIIa binding to both endothelial cell

protein C receptor and tissue factor (TF) on cell surfaces. The internalisation of PEGylated rFVIIa in endothelial cells and fibroblasts was markedly lower compared to the internalisation of rFVIIa in these cells. This group concluded that the reduced internalisation was attributed to alterations in electrostatic binding or steric hindrance as a consequence of the PEG chain and not a loss of specific receptor recognition, given that a near normal activation of factor X by glycoPEGylated rFVIIa on TF expressing cells was observed (Sen *et al.*, 2010).

In addition, Dapp *et al.* (2011) studied the PEGylation of a series of PEGylated BN(7-14) analogues (radiolabelled bombesin conjugates) for imaging and therapy in breast and prostate tumours (with molecular weights of 5 kDa, 10 kDa and 20 kDa). Incubation of PC-3 cells at 37°C with the radiolabelled PEG conjugates demonstrated cell uptake when measured at 0.25, 0.5, 1, 2, 4 and 24 hours. For each analogue, 1 µM unlabelled BN(1-14) was used to determine nonspecific binding of surface-bound and internalised activity. After 30 min of PC-3 incubation, the non-modified 99mTc-Lys-BN analogue displayed maximum internalisation, which remained stable for 2 hours. Internalisation was markedly lower with all PEGylated analogues, and the time taken to reach a plateau of internalised fraction was delayed (between 4 and 24 hours). This data was consistent with the theory of disrupted or slower binding kinetics associated with the PEGylated BN analogues compared with their non-PEGylated counterparts. The PEG5 modified BN variants displayed the most favourable *in vivo* pharmacokinetic properties such as enhanced clearance from the blood plasma and preferential excretion via the renal route. The authors also reported a  $2.9\% \pm 0.9\%$ ,  $1.7\% \pm 1.4\%$  and  $1.2\% \pm 1.2\%$  internalisation rate for the PEG5, PEG10 and PEG20- conjugated BN analogues, respectively, expressed as a percentage of total radioactivity added per million cells (% per  $10^6$  cells); although these values do not significantly alter between each other, there is evidently a trend for increased internalised fraction which is dependent upon the size of the PEG entity. To conclude, this study reported PEGylation to be an effective modification to improve the pharmacokinetic profile of novel BN analogues. The impaired intracellular uptake observed in this study also corroborates the reduced Y<sub>4</sub> receptor internalisation observed with PEGylated [K<sup>30</sup>, Q<sup>34</sup>]hPP versus other [K<sup>22</sup>, Q<sup>34</sup>]hPP peptide variants by our collaborators.



## 4.5 Lipidation of novel dual Y<sub>2</sub>/Y<sub>4</sub> and single Y<sub>4</sub> agonists

### 4.5.1 Lipidation results in protracted responses and a retention in Y<sub>4</sub> potency

The effect of N-terminal conjugation with a fatty acid moiety has been well documented; the increased stability of the secondary peptide results in increased longevity of action in comparison to the native peptide (Knudsen *et al.*, 2000; Yamamoto *et al.*, 2003). Previously, our collaborators demonstrated that palmitoylation of a hPP analogue ([K<sup>13</sup>,Q<sup>34</sup>]hPP) increased the biological activity of the peptide (Bellman-Sickert *et al.*, 2011). Bellman-Sickert *et al.* achieved *in vitro* stabilisation as well as a longer *in vivo* half-life and subsequently higher bioavailability. Furthermore, this group observed a more persistent and retarded uptake of the lipidated peptide in the rat and a re-distribution to the liver instead of the kidneys indicating a reduced risk of toxicity. Initial studies on food intake revealed that lipidation prolonged the anorectic effect compared to the unmodified analogue. Given the observation described above, the lipidated hPP analogues were further investigated as potential anti-obesity agents. The findings reported in this thesis were designed to complement this study (Bellman-Sickert *et al.*, 2011) and to determine if the introduction of an N-terminal long-chain fatty acid to the [K<sup>30</sup>, Q<sup>34</sup>]hPP and [K<sup>22</sup>, Q<sup>34</sup>]hPP variants would further enhance biological activity and potency at the Y<sub>2</sub> and Y<sub>4</sub> receptor. Furthermore, an assessment of varying fatty acid lengths on the Y<sub>4</sub>-mediated anti-secretory capabilities of the [K<sup>22</sup>]hPP<sub>2-36</sub> series of peptides was also made.

The C16 lipid-conjugated peptides, [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP, [K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP and [K<sup>22</sup>(E-Pam)]hPP<sub>2-36</sub>, demonstrated similar anti-secretory profiles, with reductions in I<sub>sc</sub> comparable to those seen with the PEGylated [K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP peptide in Col-24 monolayers. In human colon mucosa, the response to [K<sup>22</sup>(E-Pam)]hPP<sub>2-36</sub> was almost 50 % reduced compared to that seen with [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP, indicating greater efficacy with the dual Y<sub>2</sub>/Y<sub>4</sub> lipidated agonist, compared with the single Y<sub>4</sub> lipidated agonist. This result is not surprising due to the dual expression of the Y<sub>2</sub> and Y<sub>4</sub> receptor in human colonic specimens. Modification of [K<sup>22</sup>]hPP<sub>2-36</sub> with lauric acid (C12) produced equi-effective responses compared with [K<sup>22</sup>(E-Pam)]hPP<sub>2-36</sub> indicating that further elongation of the lipid side chain from C12 to C16 was not detrimental to agonist activity. Basolateral additions of [K<sup>22</sup>(E-Capr)]hPP<sub>2-36</sub> (C8) resulted in a switch from a monophasic transient profile (seen with the non-lipidated [K<sup>22</sup>]hPP<sub>2-36</sub>) to a biphasic response in epithelial monolayers, with an initial inhibitory phase that reached a nadir at 5 min followed by a second more prolonged secretory phase that lasted over

45 min (Figure 3.37). Time course profiles of the same lipidated analogues in human colon mucosa indicated a similar anti-secretory pattern to that seen in Col-24 monolayers; both  $[K^{22}(\text{E-Laur})]hPP_{2-36}$  and  $[K^{22}(\text{E-Pam})]hPP_{2-36}$  were delayed in the rate of onset compared with  $hPP_{2-36}$  and inhibited  $I_{sc}$  to the same degree. Similarly,  $[K^{22}(\text{E-Capr})]hPP_{2-36}$  displayed an initial reduction in  $I_{sc}$  followed by a second anti-secretory phase. The biphasic nature of the caprylic acid-conjugated peptide response has not been reported to my knowledge in any previous studies, however the intermediary response may be due to the length of the chain (C8); the caprylic acid conjugate may be short enough so as to not alter the binding kinetics of the peptide compared with the non-lipidated analogue,  $hPP_{2-36}$ , but may be sufficiently long to increase the overall stability and shield the core peptide from proteolytic destruction, hence resulting in a second prolonged secretory effect.

All fatty acid side chains were inactive in both colonic preparations. The fact that caprylic acid, along with lauric and palmitic acid, were all inactive (at 100 nM), confirms that the anti-secretory responses to each lipidated peptide was a result of  $Y_4$  receptor activation, and not a result of L-cell derived PYY release into the lamina propria (exerting a paracrine effect on epithelial derived  $Y_1$  actions).

Besides  $[K^{22}(\text{E-Capr})]hPP_{2-36}$ , all lipidated peptides displayed a delay in the onset of action. This characteristic latency has also been reported by a group comparing the pharmacokinetic and pharmacodynamic properties of a long-acting lipidated (C14) insulin analogue. Subcutaneous injection of NN304 into a para-umbilical skin fold of 11 healthy volunteers resulted in a predictable linear elevation in total plasma NN034 concentration, when compared with Neutral Protamine Hagedorn (NPH)-insulin during euglycaemic glucose clamp studies. Interestingly, peak concentrations were not achieved until after 4-6 hours after administration. Furthermore, the maximal glucose infusion rate observed with NN034 was not as transient compared to its non-lipidated counterpart, NPH-insulin, at an equivalent dose. NN304 also demonstrated a slower rate of onset, demonstrated by a considerably higher  $t_{max}$  (Heinemann *et al.*, 1999).

All lipidated peptides presented herein exhibited equal potency compared to the non-lipidated peptide. Additional studies examining the effects of increasing carbon side chain length on peptide activity have also revealed conserved potency after lipid conjugation. Todorovic *et al.* (2005) examined the effect of increasing the alkyl chain

length at the N-terminal by integrating long-chain fatty acids on the His-DPhe-Arg-Trp-NH<sub>2</sub> tetrapeptide, an agonist at the melanocortin receptors (MCR). A total of twelve lipidated analogues were developed to target the mouse MC<sub>1</sub>R and MC<sub>3</sub>R-MC<sub>5</sub>R, and the MC<sub>1</sub>R expressed in human melanocytes. Tetrapeptides modified with a minimum of 9 alkyl groups did not display enhanced potency at the mMC<sub>(3-5)</sub> receptors but did retain their potency compared to their non lipidated control, despite the addition of up to 16 carbons to the core peptide. In contrast, all His-DPhe-Arg-Trp-NH<sub>2</sub> tetrapeptides targeted to the mMC<sub>1</sub>R demonstrated a superior potency compared with the non-acylated control. A 20-200 fold improvement in potency was reported at the MC<sub>1</sub>R compared with the nonacylated peptide; however modification with fatty acids consisting of a minimum of eight carbons provided no additional improvement in potency at the MC<sub>1</sub>R. Interestingly, a 10, 11, 12 and 15 carbon conjugation resulted in a 10-fold reduction in potency in comparison to the 8 carbon variant, although all other lipidated tetrapeptides exhibited equal potency compared to the C8 variant. Thus, Todorovic *et al.* successfully showed enhanced potency of the tetrapeptide when conjugated to a short chain fatty acid such as the octanoyl (C8), nonanoyl (C9), and decanoyl (C10) variants, whereas although long chain fatty acid conjugation was not unfavourable in terms of potency, there was no further contribution compared with their shorter fatty acid-conjugated counterparts. Data reported by Todorovic *et al.* (2005) resembles that seen with the lipidated dual Y<sub>2</sub>/Y<sub>4</sub> and single Y<sub>4</sub> peptides presented in this study; receptor specificity (Y<sub>1</sub>) was also observed with the palmitoylated peptide, [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP (described in more detail below, section 4.5.2). Furthermore, although a 20-200 fold enhancement of potency at the MC<sub>1</sub>R was reported by Todorovic *et al.* for all 12 analogues tested, the lipidated peptides presented in this thesis all retained their potency and were equi-effective compared with the non-lipidated control, [K<sup>22</sup>]hPP<sub>2-36</sub> in Col-24 monolayers.

#### **4.5.2 Palmitoylation retains Y<sub>2</sub> and Y<sub>4</sub> activity with no significant Y<sub>1</sub> effects.**

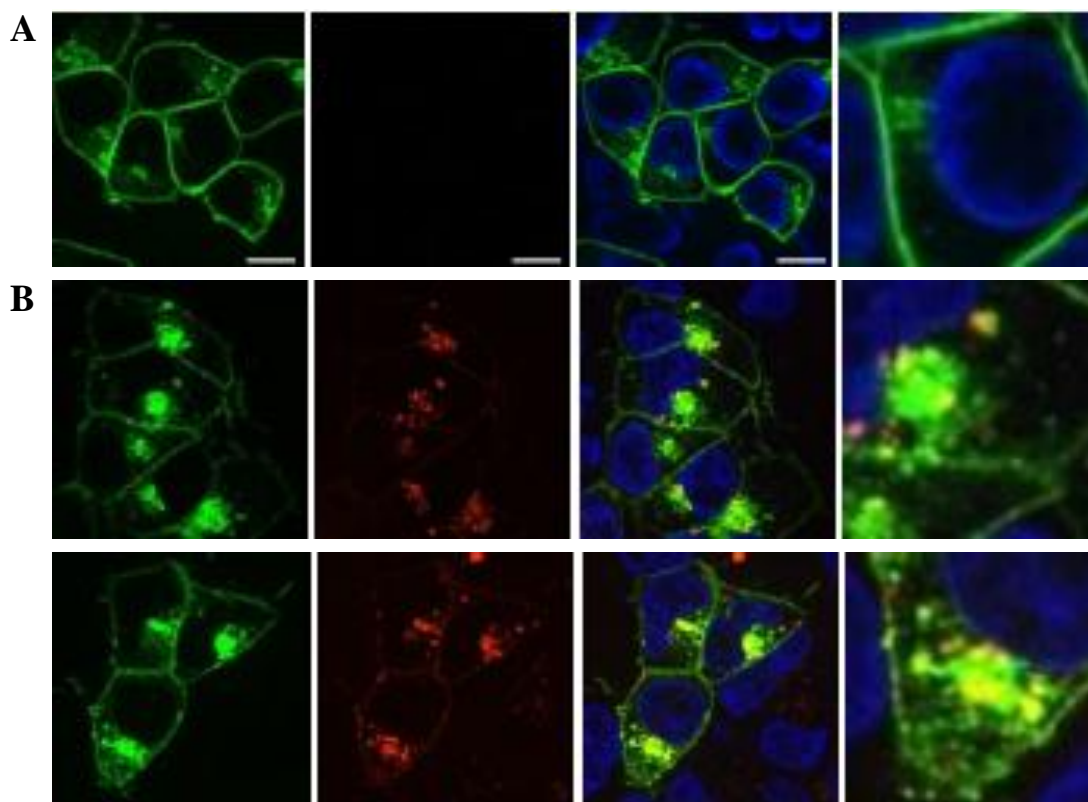
Palmitoylation of all 3 peptide series, [K<sup>30</sup>, Q<sup>34</sup>]hPP, [K<sup>22</sup>, Q<sup>34</sup>]hPP and [K<sup>22</sup>]hPP<sub>2-36</sub>, demonstrated the most promising pharmacological profiles. Concerns over nM Y<sub>1</sub> receptor affinity (albeit 2 orders of magnitude lower than the Y<sub>2</sub> and Y<sub>4</sub> for [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP, as indicated in binding studies by our collaborators, Appendix 1.0) led to use of the selective Y<sub>1</sub> (BIBO3304) to discern the role of the Y<sub>1</sub> receptor in mediating the anti-secretory effect of [K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP on the Y<sub>1</sub> (also Y<sub>2</sub> and Y<sub>4</sub>)-expressing human colonic mucosa. Here responses to the dual Y<sub>2</sub>/Y<sub>2</sub> receptor agonist were

unaltered in the presence of BIBO3304 but were significantly attenuated in the presence of BIIIE0246 (Figure 3.13); this study highlighted the selectivity of the dual agonist for the  $Y_2$  receptor over the  $Y_1$ , and since the latter is associated with the cardiovascular side effects (Malmstrom *et al.*, 2002), this makes the lipidated peptide highly desirable as a therapeutic treatment.

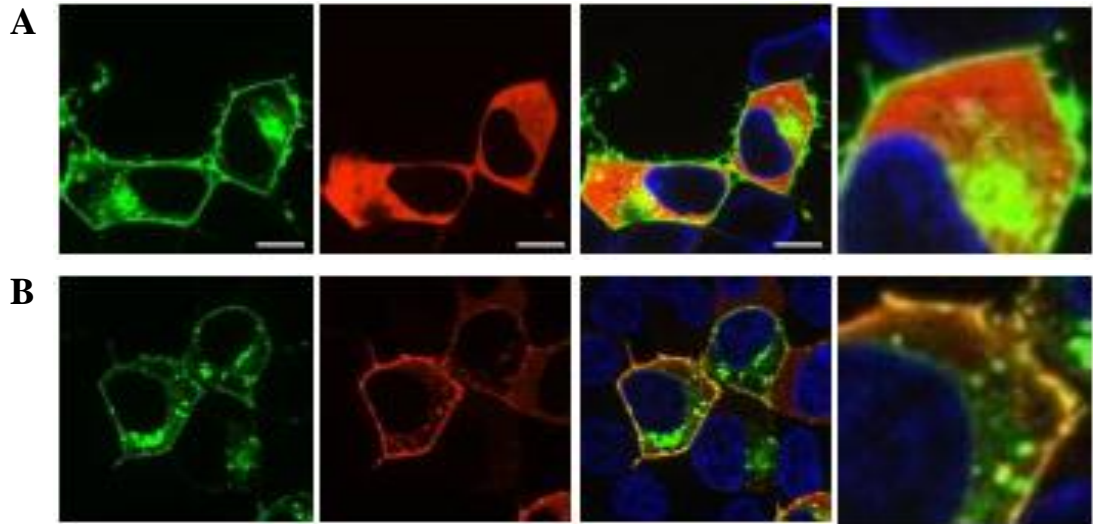
#### 4.5.3 Effect of lipidation on $Y_4$ receptor desensitisation

Contrary to the lack of  $Y_4$  receptor desensitisation observed with the PEGylated derivatives of the  $[K^{30}, Q^{34}]hPP$  and  $[K^{22}, Q^{34}]hPP$  series of peptides, lipidation of each peptide series resulted in rapid, significant desensitisation. In Col-24 monolayers, low nM concentrations of  $[K^{30}(E-Pam)Q^{34}]hPP$  significantly reduced further  $Y_4$  signalling (Figure 3.15D). Human colon mucosal pre-treatment with  $[K^{30}(E-Pam)Q^{34}]hPP$  (100 nM) also resulted in a significant reduction in the subsequent hPP responses compared with 'Cntrl' hPP (100 nM), mimicking the loss of hPP responses observed in Col-24 monolayers. Likewise, pre-treatment with  $[K^{22}(E-Pam)Q^{34}]hPP$  (0.3-100 nM) in Col-24 monolayers significantly reduced subsequent hPP responses. With regard to the fatty acid-conjugated  $[K^{22}]hPP_{2-36}$  series of peptides, the extent of  $Y_4$  receptor desensitisation positively correlated with the lipophilicity of the peptides, with palmitic acid- and lauric acid-conjugated peptides demonstrating significantly enhanced inhibition of further  $Y_4$  signalling compared with their non-lipidated predecessor.  $[K^{22}(E-Capr)]hPP_{2-36}$  also exhibited a concentration-dependent desensitisation profile which was more akin to that seen with the unmodified  $[K^{22}]hPP_{2-36}$  peptide.

This divergence in  $Y_4$  desensitisation compared with the PEGylated equivalents was also observed in imaging studies performed by our collaborators, with the use of stably transfected HEK-293 cells with EYFP- $hY_4$  receptor and co-localisation with TAMRA-modified  $[K^{22}(E-Pam)Q^{34}]hPP$ . Mäde *et al* (unpublished) demonstrated rapid internalisation of TAMRA- $[K^{22}(E-Pam)Q^{34}]hPP$  (Figure 4.5) which was also associated with intense arrestin-3 recruitment (Figure 4.6).

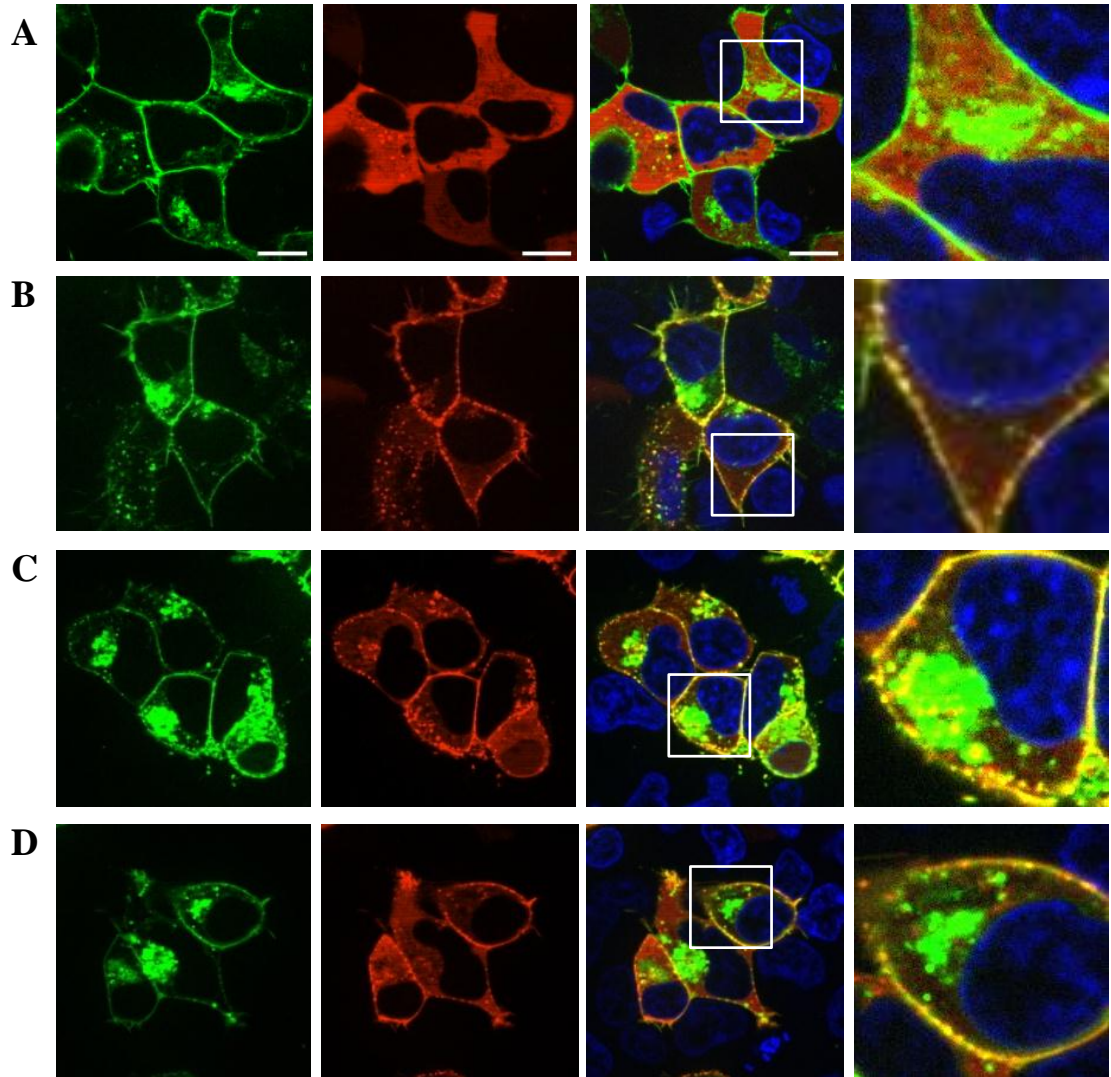


**Figure 4.5 Stimulation of HEK-293 cells expressing hY<sub>4</sub>R-EYFP co-localised with [K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP.** (A) hY<sub>4</sub>R-EYFP shown in green prior to agonist addition; TAMRA-modified peptide is shown in red, hY<sub>4</sub>R-EYFP and peptide-receptor merged (yellow) with nuclei is visualised with Hoechst33342 blue, and a 3 x magnification of a chosen cell is shown in the far right images. The scale bar represents 10  $\mu$ m. (B) Stimulation with 100 nM [K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP results in rapid and intense internalisation of the receptor-peptide complex. The upper and lower images demonstrate receptor localisation at 30 and 60 min, respectively.



**Figure 4.6 Time-dependent arrestin3-promoted hY<sub>4</sub>R internalisation upon agonist stimulation with [K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP.** (A) hY<sub>4</sub>R-EYFP and arrestin-3 localisation prior to stimulation by an agonist; with the receptor shown in green, arr-mCherry shown in red, nuclei visualised with Hoechst33342 shown in blue and a 3 x magnification shown on the right. The scale bar represents 10 µm. (B) Redistribution of arr-mCherry in response to [K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP 100 nM measured at 15 min post peptide addition.

The lipidated variants of the  $[K^{22}]hPP_{2-36}$  series,  $[K^{22}(E-Capr)]hPP_{2-36}$  and  $[K^{22}(E-Pam)]hPP_{2-36}$ , displayed intense arrestin recruitment compared with unmodified  $[K^{22}]hPP_{2-36}$  (Figure 4.7), however quantification of the amount of arrestin recruited and the time taken for recruitment is required to determine whether internalisation of the  $Y_4$  receptor is dependent on fatty acid chain length.



**Figure 4.7 Time-dependent arrestin3-promoted  $hY_4R$  internalisation upon agonist stimulation with lipidated  $[K^{22}]hPP_{2-36}$  variants.** (A) No agonist;  $hY_4R$ -EYFP and arrestin-3 localisation prior to stimulation, with the receptor shown in green (localised at the plasma membrane), arr-mCherry shown in red (ubiquitously localised in the cytoplasm), and nuclei visualised with Hoechst33342 shown in blue, and a 3 x magnification of identified individual cells (white boxes) are shown at the far right. The scale bar represents 10  $\mu m$ . Following agonist binding, redistribution of arr-mCherry is observed (measured at 15 min) in response to (B)  $[K^{22}]hPP_{2-36}$ , (C)  $[K^{22}(E-Capr)]hPP_{2-36}$  and (D)  $[K^{22}(E-Pam)]hPP_{2-36}$  (all at 100 nM), indicated by the increase in yellow decorating the membrane.

The last decade has seen the use of lipidation in drug development rise, particularly with cell penetrating peptides (CPPs); lipid tails have been conjugated to CPPs in order to enhance their membrane binding and penetration, an effect that is thought to occur through endocytosis and not a receptor-dependent mechanism. Pham *et al.* (2004) developed an amidated oligoarginine (HIV Tat peptide mimetic, R7), which was conjugated to a myristate (C14) moiety at the N-terminus. The lipoprotein was labelled with fluorescein isothiocyanate (FITC) on the C-terminal lysine side chain to monitor its cellular uptake and intracellular distribution using fluorescence microscopy and flow cytometry. Pham *et al.* demonstrated that the addition of a myristate side chain to R7-FITC significantly enhanced its association with cells and the extent of Tat peptide internalisation. However, there was little evidence of increased cytosolic labelling suggesting that the lipopeptide was simply associating with the membrane rather than translocating across it. Similar findings using myristoylated oligoarginine peptides in three different cells lines (Jurkat cells, human Tlymphoma 8E5, and myeloma U266B1 cells) were observed (Lee and Tung, 2010) and both studies demonstrated that the extent of myristoyl-mediated cell association was dependent on the length of the arginine chain. The possibility exists that the lipid aids initial membrane interaction but the lipid tail positioned in the bilayer may hinder the penetrating capacity of the peptide. Lee and Tung did however provide evidence that a myristoylated R11 peptide was able to gain access to the cytosol (Lee and Tung, 2010).

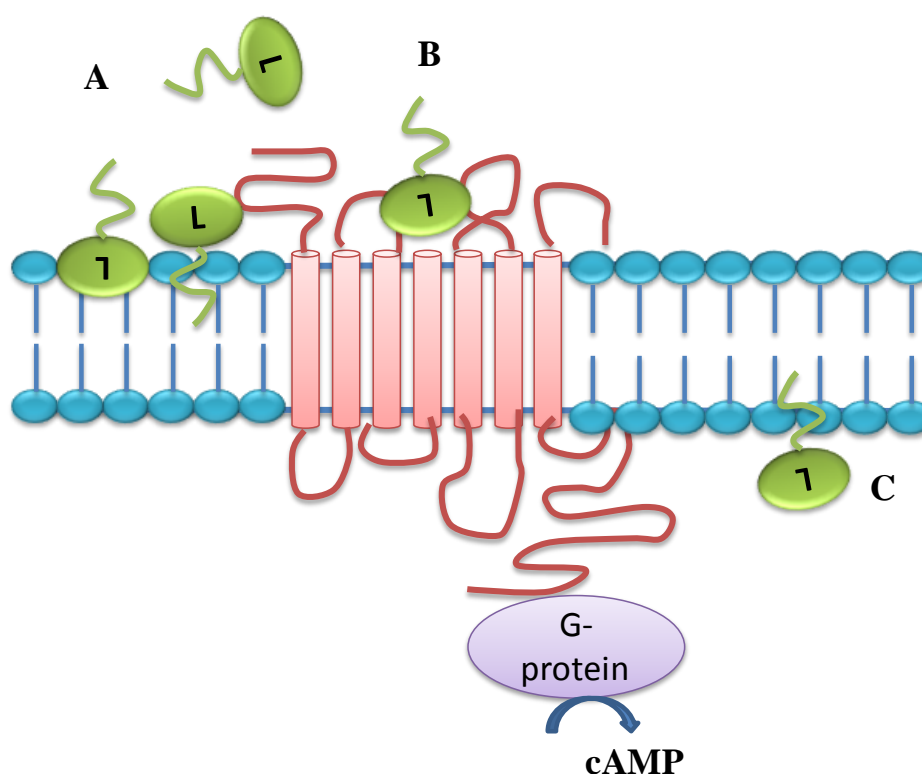
Data concerning peptide conjugation to a myristate side chain is contradictory. Two groups have demonstrated the interaction of a myristate-conjugated peptide with artificial membranes and cells and have suggested that otherwise impermeable peptides may gain access to cells via attachment to this lipid group (Ensenat-Waser *et al.*, 2002; Nelson *et al.*, 2007). Several studies have supported this notion; Fernandez-Carneado *et al.* (2005) demonstrated that fatty acylation of the N terminus of proline-rich CPPs enhance their cellular uptake into HeLa cells. Conjugating the anti-metabolite drug methotrexate with fatty acids, specifically through tris lipidation, enhances cellular uptake and leads to improved anti-tumour activity in a mouse melanoma model (Lockett *et al.*, 2000). More recently, Dudley *et al.* (2011) synthesised a series of florescent molecules, dansyl ethylenediamine and phenylalanine dansyl ethylenediamine, which were acylated with either acetate (C2), caproic (C6), enanthate (C7), myristate (C14), or palmitate (C16). Each conjugate (25  $\mu$ M) was examined for entry and localisation into cultured CHO cells by assessing total gross florescence retention and cellular



microscopic fluorescence patterns. Conjugates with myristate or palmitate demonstrated the most enhanced lipophilic property with entry into cells and localisation in the cytoplasm (Dudley *et al.* 2011). It is now widely accepted, under experimental conditions that CPPs can translocate directly across the plasma membrane of cells.

The enhanced uptake of a lipidated peptide/receptor complex through the plasma membrane is thought to occur via anchoring of the covalently linked fatty acid moiety to the membrane itself, thus facilitating rapid internalisation/translocation.

Destabilisation of the plasma membrane is considered to result from targeting of specific intracellular loops of the GPCR by the lipid moiety, enhancing ionic permeability of the bilayer. This theory has been widely acknowledged, since enhanced membrane association occurs following the addition of a hydrophobic group to a peptide (Slepnev *et al.*, 1995; Dunphy and Linder 1998; see Figure 4.8).



**Figure 4.8 Representation of the possible interaction between a lipidated ligand (L) and the Y<sub>4</sub> receptor embedded in the phospholipid bilayer.** The interaction between the long chain of the fatty acid residue and the lipid bilayer may enable ligand-receptor interaction in several steps. The ligand may accumulate at the cell surface (A) and interact with the receptor (B) with subsequent insertion into the lipid bilayer (C).

#### 4.5.4 Persistent Y<sub>4</sub> signalling despite rapid desensitisation

An interesting observation with the lipidated analogues was the continual Y<sub>4</sub>-mediated anti-secretory responses despite the apparent desensitisation of the receptor (indicated by the loss of signal to a subsequent addition of hPP). This was followed by rapid arrestin recruitment and internalisation demonstrated by the imaging data by our collaborators. Quantification of real-time cAMP generation is now possible with the utilisation of FRET tools. In 2009, two groups reported internalisation to be a prerequisite for continual G<sub>s</sub> GPCR/G-protein signalling from an intracellular endosomal compartment (Calebiro *et al.*, 2009, Ferrandon *et al.*, 2009), contradicting the classical model of GPCR signalling.

Ferrandon *et al.* studied the effects of two native ligands, parathyroid hormone (PTH) and paracrine acting PTH-related protein (PTHrP) on the signalling of the parathyroid hormone receptor (PTHR), and proposed a link between the stability of the hormone-bound receptor complex and the capacity to promote a persistent cAMP-mediated signal. The authors demonstrated conformational modifications within the G-protein and PTHR as well as cAMP activity in real time using FRET, and reported persistent receptor activation of the G-protein signal response in the presence of PTH, but not PTHrP. Stimulation with PTH also induced co-trafficking of the PTHR into early endosomes coupled with its G<sub>s</sub> protein, in contrast to PTHrP stimulation, which led to dissociation of G<sub>s</sub> protein from the PTHR, before promoting internalisation. The contradictory response to PTH and PTHrP was attributed to a stable association of the PTHR with PTH allowing a continual active conformation of the receptor-ligand complex. These data were further supported by the fact that a dynamin dominant negative mutant resulted in an inhibition in cAMP production, leading to desensitisation of the PTHR receptor (Ferrandon *et al.*, 2009). The authors concluded that a protective mechanism was involved; uptake into an endosomal compartment may shield the receptor-hormone complex from  $\beta$ -arrestin-facilitated desensitisation, thus allowing sustained GPCR signalling.

In 2009, Calebiro *et al.* supported these findings by reporting rapid internalisation of the thyroid stimulating hormone receptor (TSHR) coupled with its associated G<sub>s</sub> protein and AC after stimulation with thyroid stimulating hormone (TSH). In addition, the TSH-induced cAMP signal remained persistent and did not desensitise. Interestingly, the authors concluded that the process of internalisation prohibited receptor-hormone

dissociation since sustained cAMP generation was not observed with transient TSH stimulation, only with prolonged stimulation. As observed with the TSHR, internalisation of the PTHR–PTH complex was not associated with desensitisation of the cAMP response but rather with persistent signalling.

Evidence provided by these two groups supports the idea of persistent signalling to AC by internalised GPCRs and may help elucidate why prolonged Y<sub>4</sub>-mediated anti-secretory signals have been observed to chemically modified hPP analogues in this study. A possible mechanism for the differential Y<sub>4</sub> responses observed with the modified peptides could be attributed to a stable association of the Y<sub>4</sub> receptor/peptide complex in a prolonged active conformation. The lipid/PEG moiety may serve to position the core peptide into a conformation which has either a high or low association with arrestin, but permits the internalisation of the complex in the presence of the lipid, allowing continual receptor signalling. Thus, a possible explanation for the loss in subsequent hPP responses in Col-24 monolayers and human colon mucosa may be due to a loss in total cell surface receptor expression, rather than desensitisation of the receptor. This would explain why anti-secretory responses continue 45 min after the addition of a lipidated peptide, but responses to hPP are lost in this time period. In keeping with these findings, rapid internalisation was observed for all lipidated analogues in imaging experiments. Although fast arrestin recruitment was also measured, it would now be interesting to quantify the extent of arrestin-mediated phosphorylation of the receptor, compared with the native hormone.

Whilst these two reports provide strong evidence for the continuation of G<sub>s</sub>-mediated GPCR signalling in endosomal compartments, an additional study reported intracellular G<sub>i</sub>-dependent signalling; FTY720, an immunomodulator drug used in the treatment of multiple sclerosis, predominately targets the G<sub>i</sub>/ G<sub>q</sub>-coupled sphingosine-1-phosphate receptor 1 (S1P1) (Kappos *et al.*, 2006). Upon binding to the receptor (Mandala *et al.*, 2002), the receptor-ligand complex undergoes endocytosis from the cell surface (Oo *et al.*, 2007). Mullershausen *et al.* demonstrated inhibition of forskolin-induced cAMP production 5 hours post internalisation in addition to MAPK activation, despite almost complete co-localisation of the S1P1 receptors within vesicular compartments at the same time point (Mullershausen *et al.*, 2009). Furthermore, internalisation of the receptor resulted in a loss of IP<sub>3</sub> signalling, which is in accordance with the view that generation of IP<sub>3</sub> takes place solely at the plasma membrane. One explanation for this

observation is that internalisation occurs with the ligand-GPCR-AC complex as one macromolecular entity. Whilst the ligand is still bound to the receptor, the complex remains active. Although the functional data presented in this thesis along with the imaging data performed by our collaborator does not provide direct proof to support the inhibition of cAMP via the Y<sub>4</sub> receptor, these results do give an indication for the existence of persistent Y<sub>4</sub> receptor signalling. To the best of my knowledge, all studies to date to monitor persistent cAMP signalling by GPCRs have been performed in cells in tissue culture. Thus, the physiological significance of persistent cAMP signalling by GPCRs is yet to be elucidated.

#### **4.6 Current pharmacotherapies in development for obesity and the potential of Obinepitide and TM30339 analogues**

While there is extensive evidence that points to the acute anorexigenic effects of both the Y<sub>2</sub> and Y<sub>4</sub> agonists, the rapid degradation of PYY and PP, and short circulating half-lives, imply that use of the native peptides may not be so successful with respect to chronic treatment. Moreover, PYY(3-36) or PP administered to the CNS of rodents has been shown to promote appetite (Clark *et al.*, 1987; Asakawa *et al.*, 1999; Corp *et al.*, 2001). This is thought to be due to high local concentrations of these hormones pharmacologically stimulating orexigenic Y<sub>1</sub> and Y<sub>5</sub> receptors. In addition, the therapeutic window for PYY(3-36) is relatively narrow, with low doses proving ineffective and high doses causing nausea (Degen *et al.*, 2005).

A number of endogenous hormones implicated in metabolic conditions have undergone successful PEGylation to enhance their bioavailability, including GLP-1 (Lee *et al.*, 2005; Lee *et al.*, 2006), calcitonin (Shin *et al.*, 2004), insulin (Shechter *et al.*, 2008) and NPY (Ortiz *et al.*, 2007). In the case of PEG-calcitonin, Shin *et al.* attempted to reduce the rate of administration by improving the pharmacokinetic–pharmacodynamic profile; the 2kDa PEG moiety of the polypeptide promoted slower absorption by reducing the permeability across the nasal epithelium (Shin *et al.*, 2004). However, not all intranasal formulations have been successful; pre-prandial intranasal PYY(3-36) was tested in obese patients for two weeks, but was not found to reduce body weight. Intranasal administration resulted in transiently high circulating levels of PYY associated with nausea (Gantz *et al.*, 2007). PYY analogues which show moderately increased but persistent circulating levels following administration may be more useful in the treatment of obesity. A PEGylated selective Y<sub>2</sub> receptor agonist has been shown to

outperform PYY(3-36) in reducing food intake in mice (DeCarr *et al.*, 2007), suggesting that PEGylation of specific Y<sub>2</sub> receptor agonists may improve their therapeutic potential.

The GIPIO collaboration aimed to address these shortcomings above by developing more stable, longer acting and highly selective analogues. By developing a hPP analogue with additional Y<sub>2</sub> affinity, as is the case of Obinipitide ([Q<sup>34</sup>]hPP), fewer side effects were predicted compared with selective Y<sub>2</sub> analogues (see section Figure 4.7). Obinipitide has been shown to inhibit food intake in obese individuals up to nine hours after subcutaneous administration in phase I/II trials (7TM Pharma, 2008). In addition, recent developments of PEGylated or fatty acid acylated Obinipitide variants have demonstrated improved pharmacokinetic properties compared to a non-modified human PP analogue (Bellmann-Sickert *et al.*, 2011), and for these reasons, the novel peptides assessed in this study deserve further investigation into their potential as anti-obesity therapies. Likewise, TM30339 (hPP<sub>2-36</sub>) proved efficacious in diet-induced obese mice by reducing body weight in phase I/II trials (7TM Pharma, 2008) and thus the sustained functional Y<sub>4</sub> responses and high potency observed with the palmitic acid/lauric acid-conjugated variants makes a stronger case for *in vivo* studies with the lipidated peptides.

#### **4.7 Peripheral Y<sub>2</sub> and Y<sub>4</sub> mechanisms and potential side effects**

In parallel with their central anorexigenic activities, both the Y<sub>2</sub> and Y<sub>4</sub> receptors exhibit multiple peripheral mechanisms via PP, PYY and PYY(3-36); several of these mechanisms are inhibitory and play a pivotal role in the regulation of digestive processes, including inhibition of electrolyte secretion (Playford *et al.*, 1990; Cox and Tough, 2002), reduction in gastric acid secretion, slowing of gastric emptying and upper GI transit, and slowing of colonic motility, working in combination to modulate food intake and energy expenditure.

Receptor-mediated colonic absorption via a combination of Y<sub>1</sub>, Y<sub>2</sub>, or Y<sub>4</sub> receptors in human specimens occurs as a result of attenuated apical Cl<sup>-</sup> secretion, although Y<sub>5</sub> agonists are ineffective within the GI tract (Cox *et al.*, 2001a; Cox and Tough, 2002). Since chemically modified PEGylated or lipidated Y<sub>2</sub>/Y<sub>4</sub> or single Y<sub>4</sub> agonists consistently demonstrated prolonged reductions in I<sub>sc</sub> in isolated human mucosa, there is a potential for unwanted side effects such as reduced ion and electrolyte secretion and hence constipation. The functional observations in this study provide a strong justification for the dual agonists to be used not only as anti-obesity treatments, but also

in conditions such as severe diarrhoea (since CFTR-mediated  $\text{Cl}^-$  secretion in the intestine is accountable for a number of secretory diarrhoeas). The long-acting selective  $\text{Y}_4$  agonists (PEG/lipid- $[\text{K}^{22}]\text{hPP}_{2-36}$ ) offer further advantages to clinical treatment, due to the limited peripheral localisation of the  $\text{Y}_4$  receptor (in contrast to the extensive distribution of  $\text{Y}_1$  and  $\text{Y}_2$  receptors).

As well as their fundamental importance in gut in terms of electrolyte absorption, several studies have now documented profound inhibitory effects of both the  $\text{Y}_2$  and  $\text{Y}_4$  (and  $\text{Y}_1$ ) receptors on gastric acid secretion and upper GI motility. Field *et al* recently reported an inhibition of both gastric emptying and gastric acid secretion, and an overall reduction in the mouth-to-caecum transit time following *i.v.* infusion of PYY in humans (Field *et al.*, 2010). The inhibitory effect on gastric secretion is thought to be  $\text{Y}_1$  and  $\text{Y}_2$ -mediated through actions in brainstem and stomach (Yang, 2002). With respect to upper small intestinal transit, PYY acts to slow transit by inducing an ‘ileal brake’ mechanism (Lin *et al.*, 1996; Maljaars *et al.*, 2008), contributing to satiation. These findings have been supported by Tough *et al.* (2011), who recently demonstrated significantly increased transit in PYY $^{-/-}$  mice compared with WT mice. A slightly increased upper GI transit following *i.p.* administration of BIIE0246 to WT mice was also observed, suggesting that the slowing of transit by endogenous PYY occurs through peripheral  $\text{Y}_2$  and possibly  $\text{Y}_1$  receptors. Interestingly, the authors demonstrated an equal basal rate for gastric emptying in both PYY and NPY KO mice compared to WT mice; Tough *et al.* suggested this could be attributed to the lack of PYY’s peripheral mechanism to inhibit upper intestinal transit, coupled with NPY’s ability to stimulate gastric emptying through a central pathway (via activation of the dorsal vagal complex; Mönnikes *et al.*, 2000), thus counteracting each other. Limited data exist for the effects of PP on gastric emptying. Early reports suggested the inability of bPP to inhibit gastric emptying in human subjects (Adrian *et al.*, 1981), although other groups successfully went on to demonstrate an inhibitory role of the native peptide in rats (Okumura *et al.*, 1994), mice (Asakawa *et al.*, 2003) and humans (Schmidt *et al.*, 2005), which was recently confirmed to be dependent on intact vagal signalling (Field *et al.*, 2010). A considerable amount of evidence cumulatively indicates that rapid gastric emptying is strongly related to increased food consumption and obesity, whereas a delay in emptying is seen in patients with conditions such as cachexia and anorexia (Duggan and Booth, 1986; Inui, 1999; Inui, 2002). For this reason it is predicted that the novel PEG/lipid dual

analogues may illicit further anorexigenic actions *in vivo*, by inducing a Y<sub>2</sub>/Y<sub>4</sub>-mediated slowing of gastric emptying.

Exogenous NPY, PYY and PP can evoke Y<sub>2</sub> and Y<sub>4</sub> receptor-mediated contractility of colonic smooth muscle (Pheng *et al.*, 1999; Hyland *et al.*, 2003). In 2010, Wang *et al.* demonstrated sustained inhibitory effects on basal- and stress-stimulated colonic propulsive motor function following *i.p.* administration of PYY(3–36) or PYY in conscious mice, an effect which was Y<sub>2</sub> receptor-mediated (Wang *et al.*, 2010). Tough *et al.* (2011) corroborated these findings by reporting that the slowing of colonic transit was dependent upon Y<sub>2</sub> activation, via PYY and NPY. The authors reported a Y<sub>2</sub>-mediated contraction of smooth muscle (via PYY, inhibited by a Y<sub>2</sub> antagonist), thought to be a consequence of inhibition of tonic drive to the longitudinal muscle. PYY's tonic role in longitudinal muscle contraction is highlighted by the explosive diarrhoea observed in mice following administration of BIIE0246 (Wang *et al.*, 2010). Several studies have revealed the role of PP in inducing longitudinal smooth muscle contractions in colonic tissue from mice (Hyland *et al.*, 2003) and rats (Feletou *et al.*, 1998; Ferrier *et al.*, 2000; Pheng *et al.*, 1999). In 2010, Moriya *et al.* demonstrated a dose-dependent increase in faecal weight following peripheral administration of PP and almost complete inhibition of PP-induced faecal output in Y<sub>4</sub><sup>-/-</sup> mice, suggesting PP enhances colonic transit via the Y<sub>4</sub> receptor. With the use of RT-PCR, Ferrier *et al.* established high levels of Y<sub>4</sub> receptor expression within the muscle layers of the colon, particularly within the proximal and distal colon (Ferrier *et al.*, 2002). Lower expression levels however were found within the mucosa, indicating that Y<sub>4</sub> receptors within the colonic muscle layers are likely to be involved in colonic transit, whereas mucosal Y<sub>4</sub> receptors most likely play a key role in modulating colonic ion absorption *in vivo*.

Unfortunately, the therapeutic significance of PYY(3-36) is limited by the nausea and vomiting induced by exogenous administration of the peptide, predominantly at higher doses (Gantz *et al.*, 2007; le Roux *et al.*, 2008). Le Roux *et al.* reported nausea in human subjects following *i.v.* PYY(3-36) at super-physiological doses, although no further reductions in food intake were observed (le Roux *et al.*, 2008). Here the authors suggested that PYY(3-36) can decrease the amount of food intake without the onset of nausea, however reaching a certain threshold concentration may induce nausea but with no additional decrease in food intake. In contrast to PYY, nausea is not observed in human subjects following administration of PP, despite reductions in food intake

(Batterham *et al.*, 2003; Jesudason *et al.*, 2007; le Roux *et al.*, 2008), making PP analogues more attractive from a clinical perspective. Promising data published by Tan *et al.* (2012) reported no adverse events or any clinical concern when monitoring the tolerability of the novel Y<sub>4</sub> peptide, PP 1420, in healthy human volunteers. PP 1420 exhibited comparable results to the placebo with regards to changes in vital signs, haematology, biochemistry, urinalysis and ECG readings. Additionally, a visual analogue scales (VAS) assessment, used to measure the grading of nausea severity, indicated no significant level of nausea following administration of the novel Y<sub>4</sub> analogue (Tan *et al.*, 2012).

Since prolonged anti-secretory responses have been observed with both PEGylated and lipidated Y<sub>2</sub>/Y<sub>4</sub> and single Y<sub>4</sub> peptide variants *in vitro*, *in vivo* evaluation will be essential in understanding the potential adverse effects associated with the peptides. Although high doses of the gut hormones may cause aversive effects, it is predicted that administration of lower doses of gut hormones in combination may limit the likelihood of undesirable effects such as nausea, yet provide a synergistic action on the inhibition of food intake. To circumvent the weak but significant interaction of PYY(3-36) and PP with the orexigenic Y<sub>1</sub> and Y<sub>5</sub> receptors (Gerald *et al.*, 1996 and Kanatani *et al.*, 2000, respectively), a combination of the dual Y<sub>2</sub>/Y<sub>4</sub> analogue along with Y<sub>1</sub> or Y<sub>5</sub> receptor antagonist may further increase the overall anorexigenic effect. Furthermore, signalling data provided by our collaborators has shown weak interaction of these peptides with both the Y<sub>1</sub> and Y<sub>5</sub> receptor (see Appendix 1.0), making these peptide clinically far more desirable.

Modulating gut hormones to control appetite is still under-developed as a pharmacotherapy, although the proof-of-concept is provided by the success of GLP-1 therapy, for patients with type 2 diabetes. Despite numerous studies demonstrating the use of GLP-1 in the treatment of type 2 diabetes, the endogenous peptide undergoes rapid degradation by DPP-IV, thus resulting in the need for continuous subcutaneous injection for clinical efficacy (Drucker and Nauck, 2006). Liraglutide, the human GLP-1 analogue, is a glucose-lowering agent with a significantly prolonged action compared with the native GLP-1 which has been achieved through fatty acid acylation. Liraglutide exhibits increased affinity for blood albumin and therefore allows once-daily subcutaneous administration. In the UK, liraglutide 1.2 mg/day now represents an additional therapy widely available for very obese (BMI  $\geq 35.0$  kg/m<sup>2</sup>) patients or for



those unsuitable for insulin and are failing to meet targets for glycaemic control with oral agents (NICE technology appraisal guidance, 2010). While this emphasises the importance of suppressing weight gain in the treatment of type 2 diabetes, the only class of currently available clinically applicable drugs that induce weight loss are GLP-1 receptor agonists. Thus, the chemically modified gut hormones in this thesis present a promising opportunity for anti-obesity pharmacotherapy and with restricted localised peripheral and central Y receptor targets, few nonspecific side effects are predicted.

#### 4.8 Concluding statement

Due to the side effects and limited efficacy of the anti-obesity mono-therapies currently available, there is an urgent need for a dual treatment with enhanced efficacy, selectivity and sustained activity. The GIPIO collaboration has aimed to address this problem by developing dual agonists that exhibit both Y<sub>2</sub> and Y<sub>4</sub> anorexigenic properties, in addition to a range of selective Y<sub>4</sub> analogues with improved *in vitro* and *in vivo* characteristics over the native hormone, hPP. The results herein provide preclinical evidence in two human preparations that PEG- and lipid-modified PP analogues retain full potency and display enhanced *in vitro* characteristics. More specifically, it suggests that PEG5-ylation and palmitoylation of the core peptide sequences, [K<sup>22</sup>, Q<sup>34</sup>]hPP and [K<sup>30</sup>, Q<sup>34</sup>]hPP have well-preserved dual receptor biological activity as compared with hPP. The anti-secretory effects of single Y<sub>4</sub> lipidated agonists, based on the hPP<sub>2-36</sub> parent peptide, were also studied with an emphasis on the effects of changes in the fatty acid chain length on *in vitro* activity. The agonists were additionally well tolerated in terms of *in vitro* properties, with long chain fatty acid modification causing a predictable increase in longevity of action. Since the Y<sub>4</sub> receptor has a more discrete peripheral localisation than Y<sub>1</sub> or Y<sub>2</sub> receptors, the limited expression is beneficial in terms of drug development. As well as the potential as anti-obesity benefits, selective Y<sub>4</sub> receptor agonists could also be used to modulate GI function with limited side-effects compared with agents targeting the more ubiquitous Y<sub>1</sub> and Y<sub>2</sub> receptors. For the treatment of conditions responsive to Y<sub>4</sub> agonism, such as obesity and intestinal hyper-secretion, it would be more desirable to use longer-acting Y<sub>4</sub> receptor selective agonists.

The results in this study demonstrated a resistance to Y<sub>4</sub> receptor desensitisation when stimulated with a PEGylated analogue and a contradictory enhancement of desensitisation following stimulation with a lipidated analogue. This divergence was further supported by fluorescence imaging studies; our collaborators demonstrated that

the ligand-bound Y<sub>4</sub> receptor remained anchored to the plasma membrane following activation by PEGylated peptides and in contrast was rapidly internalised following activation with lipidated peptides. Taken together, these data suggest that a chemically modified agonist's *in vitro* kinetics and efficacy differentially modulate the mechanism of receptor-mediated endocytosis. The physiological regulation of GPCRs (through desensitisation and internalisation) may influence the clinical development of tolerance and dependence in response to chronic drug treatment. For example, the lack of desensitisation with a PEGylated peptide could be clinically desirable due to the lack of tolerance predicted with these analogues, whereas the rapid loss of Y<sub>4</sub> receptor responsiveness with a fatty acid conjugated peptide may be less predictable due to the possibility of rapid removal of the receptor from the plasma membrane but persistent signalling of internalised Y<sub>4</sub> receptors. Further investigations are warranted to elucidate the life cycle of the Y<sub>4</sub> receptor; membrane targeting, ligand binding, signalling, internalisation, recycling and degradation will all help provide new insights into the mechanism of receptor signalling in the presence of a chemically modified ligand. Furthermore, it is predicted that the selective activation of both the Y<sub>2</sub> and Y<sub>4</sub> receptors have promising potential for the treatment of obesity and the results consequently merit the investigation of these peptides in humans to establish their clinical efficacy and likelihood of adverse effects, under physiological and pathological conditions.

## **APPENDIX**

## Peptide synthesis

Peptide analogues were synthesised by solid-phase peptide synthesis (SPPS). For synthesis, the Rink amide resin (4-(20,40-dimethoxyphenyl)-fluorenylmethoxy carbonyl-aminomethyl-phenoxyacetoamido-norleucylaminomethyl) was used in order to obtain an amidated C-terminus as already described in the literature (Rennert et al., 2006). Site-specific modification by PEG2, PEG5, PEG22 and palmitic acid was achieved at the N<sup>ε</sup>-amino group of Lys at position 22 or 30. This group could specifically be removed by hydrazine, thereby releasing the free amino group. To prevent a loss in affinity and to increase water solubility, the Pam group was linked to the peptide by a glutamyl spacer, coupled to Lys<sup>30</sup> via the  $\gamma$ -carboxyl group of an N-terminally fluorenylmethoxycarbonyl (Fmoc) and C-terminally tert-butyl (tBu) protected Glu. PEG-polymers of either 2 kDa, 5 kDa or 22 kDa were selectively incorporated at a lysine side chain in solution using a photocleavable protecting group at the N-terminus which was then removed after PEGylation. Modified peptides were characterised by reversed-phase HPLC and mass spectrometry. Purification was carried out by RP-HPLC.

## Agonist induced IP<sub>3</sub> generation

Potency of the analogues on each human Y receptor type was determined by performing concentration-response experiments in COS-7 cells transiently transfected with the Y receptor as well as a promiscuous G-protein, p7TM678. This ensured that the Y receptor coupled through a G<sub>q</sub> pathway leading to an increase in IP<sub>3</sub> turnover. One day after transfection COS-7 cells were transferred to 96-well culture plates at a density of 30,000 cells per well and incubated for 24 hours with 0.5  $\mu$ Ci of [<sup>3</sup>H]-myo-inositol in 100  $\mu$ l medium supplemented with 10% FCS, 2 mM glutamine and 0.01 mg/ml gentamicin per well. Cells were washed twice in buffer, 20 mM HEPES, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 10 mM glucose, 0.05% (w/v) bovine serum; and incubated in 100  $\mu$ l buffer supplemented with 10 mM LiCl at 37° C for 30 min. After stimulation with various concentrations of peptide for 60 min at 37° C, cells were extracted with 50  $\mu$ l 10% ice-cold perchloric acid followed by incubation on ice for 30 min. 20  $\mu$ l of the perchloric acid cell solution was transferred into a 96 wells plate. 80  $\mu$ l of SPA YSI beads (12.5 mg/ml) (=1 mg/well) was added, the plate was sealed and shaken up to 30 min. After centrifugation for 5 min at 1500 rpm, the amount of radioactive phosphoinositide produced in response to increasing concentrations of agonist was analysed with a Packard Topcounter and

subsequently led to the generation of EC<sub>50</sub> values for the [K<sup>30</sup>,Q<sup>34</sup>]hPP series (Table 1.0), [K<sup>22</sup>,Q<sup>34</sup>]hPP series (Table 2.0) and the hPP<sub>2-36</sub> series of peptides (Table 3.0).

## **Y<sub>4</sub> receptor imaging studies**

### **Cell culture**

HEK293 cells stably transfected with the human Y<sub>4</sub>-receptor and C-terminally fused to eYFP (HEK293-hY<sub>4</sub>R-eYFP) as described in Böhme *et al.* 2008 were cultured in DMEM/Ham's F-12 (1:1) without L-glutamine containing 15% (v/v) heat-inactivated fetal calf serum FCS and 100 µg/mL hygromycin B. Cells were grown as monolayers and grown to confluence at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### **Fluorescence microscopy**

For live cell imaging experiments cells were seeded into sterile µ-Slides 8 well (ibidi GmbH, Martinsried, Germany) and cultured to 80 % confluency. Transient cell transfection was performed using up to 0.9 µg hY<sub>4</sub>-eYFP-pVito2 plasmid-DNA and 0.1 µg of a β-arrestin3-mCherry construct (Walther *et al.* 2010) and up to 2 µl Lipofectamine™ 2000 Transfection Reagent. Prior to ligand stimulation, cells were starved in Opti-MEM® reduced serum medium for 30 min and stimulated with 100 nM of either fluorescent-labeled peptide or non-fluorescent labeled conjugates in Opti-MEM® reduced serum medium at 37 °C for different time periods (10 min, 30 min or 60 min). Stimulation was terminated by washing the cells with Hanks' balanced salt solution (HBSS) and cells were imaged. The cell nuclei were visualized with Hoechst 33342 (1 µg/ml; Sigma-Aldrich, Taufkirchen, Germany) for 10 min at room temperature. Glass cover slips were mounted on microscope slides with FluoromountG (Carl Roth GmbH & Co. KG, Wiesbaden, Germany). Fluorescence images from living cells were obtained with an Axio Observer microscope equipped with an ApoTome imaging system and a Heating Insert P Lab-Ek S1 unit (Zeiss, Jena, Germany). Apotome images were collected using the AxioVision software (Release 4.8) at room temperature.

	IP <sub>3</sub> Y <sub>1</sub> (nM)	IP <sub>3</sub> Y <sub>2</sub> (nM)	IP <sub>3</sub> Y <sub>4</sub> (nM)	IP <sub>3</sub> Y <sub>5</sub> (nM)	Receptor Preference
<b>hPP</b>	170.0	9100	0.6	30.0	Y <sub>4</sub> >Y <sub>5</sub> >Y <sub>1</sub> >Y <sub>2</sub>
<b>[Q<sup>34</sup>]hPP</b>	678.0	2.2	0.9	19.0	Y <sub>4</sub> >Y <sub>2</sub> >Y <sub>5</sub> >Y <sub>1</sub>
<b>[K<sup>30</sup>(PEG2)Q<sup>34</sup>]hPP</b>	483.0	8.4	25.3	1100	Y <sub>2</sub> >Y <sub>4</sub> >Y <sub>1</sub> >Y <sub>5</sub>
<b>[K<sup>30</sup>(PEG5)Q<sup>34</sup>]hPP</b>	> 10,000	33.0	740.0	> 10,000	Y <sub>2</sub> >Y <sub>4</sub> >Y <sub>1</sub> >Y <sub>5</sub>
<b>[K<sup>30</sup>(PEG22)Q<sup>34</sup>]hPP</b>	nd	nd	nd	nd	-
<b>[K<sup>30</sup>(E-Pam)Q<sup>34</sup>]hPP</b>	46.2	0.5	1.0	20.8	Y <sub>2</sub> >Y <sub>4</sub> >Y <sub>5</sub> >Y <sub>1</sub>

**Table 1.0 EC<sub>50</sub> values generated by an IP<sub>3</sub> accumulation assay, induced by chemically modified [K<sup>30</sup>,Q<sup>34</sup>]hPP derivatives.** Potency and selectivity of the peptide analogues with their subsequent Y receptor preferences, determined by an IP<sub>3</sub> turnover assay. Experiments were performed with COS-7 cells transiently transfected with the human Y receptor type. nd: not detectable

Peptide	IP <sub>3</sub> Y <sub>1</sub> (nM)	IP <sub>3</sub> Y <sub>2</sub> (nM)	IP <sub>3</sub> Y <sub>4</sub> (nM)	IP <sub>3</sub> Y <sub>5</sub> (nM)	Receptor Preference
<b>[K<sup>22</sup>,Q<sup>34</sup>]hPP</b>	>1000	0.2	0.5	3.8	Y <sub>2</sub> >Y <sub>4</sub> >Y <sub>5</sub> >Y <sub>1</sub>
<b>[K<sup>22</sup>(PEG5)Q<sup>34</sup>]hPP</b>	> 1000	12.0	3.0	199	Y <sub>4</sub> >Y <sub>2</sub> >Y <sub>5</sub> >Y <sub>1</sub>
<b>[K<sup>22</sup>(PEG22)Q<sup>34</sup>]hPP</b>	>1000	36.0	14.0	>1000	Y <sub>4</sub> >Y <sub>2</sub> >Y <sub>5</sub> >Y <sub>1</sub>
<b>[K<sup>22</sup>(E-Pam)Q<sup>34</sup>]hPP</b>	16.0	0.1	1.0	1.5	Y <sub>2</sub> >Y <sub>4</sub> >Y <sub>5</sub> >Y <sub>1</sub>

**Table 2.0 EC<sub>50</sub> values generated by an IP<sub>3</sub> accumulation assay, induced by chemically modified [K<sup>22</sup>,Q<sup>34</sup>]hPP derivatives.** Potency and selectivity of the peptide analogues with their subsequent Y receptor preferences, determined by an IP<sub>3</sub> turnover assay. Experiments were performed with COS-7 cells transiently transfected with the human Y receptor type.

Peptide	IP <sub>3</sub> Y <sub>1</sub>	IP <sub>3</sub> Y <sub>2</sub>	IP <sub>3</sub> Y <sub>4</sub>	IP <sub>3</sub> Y <sub>5</sub>	Receptor preference
<b>hPP<sub>2-36</sub></b>	232.0	303.0	1.0	7.9	Y <sub>4</sub> >Y <sub>2</sub> >Y <sub>5</sub> >Y <sub>1</sub>
<b>[K<sup>22</sup>]hPP<sub>2-36</sub></b>	60.0	63.0	1.9	5.1	Y <sub>4</sub> >Y <sub>5</sub> >Y <sub>1</sub> >Y <sub>2</sub>
<b>[K<sup>22</sup>(E-Capr)]hPP<sub>2-36</sub></b>	636.0	nd	3.7	nd	Y <sub>4</sub> >Y <sub>1</sub>
<b>[K<sup>22</sup>(E-Laur)]hPP<sub>2-36</sub></b>	214.0	nd	1.5	nd	Y <sub>4</sub> >Y <sub>1</sub>
<b>[K<sup>22</sup>(E-Pam)]hPP<sub>2-36</sub></b>	17.0	0.3	1.9	nd	Y <sub>4</sub> >Y <sub>1</sub>
<b>[K<sup>22</sup>(PEG22)]hPP<sub>2-36</sub></b>	52.0	>1000	5.3	583.0	Y <sub>4</sub> >Y <sub>2</sub>

**Table 3.0 EC<sub>50</sub> values generated by an IP<sub>3</sub> accumulation assay, induced by chemically modified hPP<sub>2-36</sub> derivatives.** Potency and selectivity of the peptide analogues with their subsequent Y receptor preferences, determined by an IP<sub>3</sub> turnover assay. Experiments were performed with COS-7 cells transiently transfected with the human Y receptor type. nd: not detectable.

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